Analysis of the expression and function of the *homothorax* isoforms in *Drosophila melanogaster*

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Analysis of the expression and function of the *homothorax* isoforms in *Drosophila melanogaster*

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ABSTRACT

In *Drosophila melanogaster* the gene *homothorax* (*hth*) plays many important functions during development. It encodes for a homeodomain protein belonging to the TALE-homeodomain ('three amino acid loop extension') family of conserved transcription factors. Hth interacts with Extradenticle (Exd), another member of the TALE family.
Here I analyze the functions of *hth* and its relationship with *exd*. Hth fulfils many important different functions during embryonic and larval development in *Drosophila*, which encompass from subdivision and specification of body parts to assembly of heterochromatin. The many different functions described for Hth rely on the complexity of its *locus*, from which six different isoforms arise.
I report that the distinct Hth isoforms have different expression patterns and function differentially in embryonic development. Moreover, not all the isoforms are able to translocate its partner Exd into the nucleus, and the FlHth activates *exd* transcription. The high levels of Exd protein facilitate its entrance to the nucleus.
This work demonstrates that *hth* is a complex gene that should not be considered as a functional unit. The roles of the different isoforms probably rely on their distinct protein domains and conformations and, at the end, on interactions with particular partners.

Abbreviation list

A/P anteroposterior
abd-A abdominal-A
Abd-B Abdominal-B
bab bric a brac
BSA bovine serum albumine
ceh-20 *C. elegans* homeobox-20
exd extradenticle
Flhth full-length homothorax
HD homeodomain
HM homothorax/meis domain
hth homothorax
meis myeloid ecotropic insertion site
ML-DmD8 dorsal mesothoracic disc cell line
NES nuclear export signal
OE overexpression
pbx pre-B-cell leukemia homeobox
RLS restless leg syndrome
S2 Schneider 2 cell line
sal spalt
TALE three aminoacids loop extension
Ubx ultrabithorax
unc-62 uncoordinated-62

I. INTRODUCTION

INTRODUCTION

I1 The developmental biology

The developmental biology studies the formation of an organism, from fecundation until the formation of the adult.

Actually, once reached the adulthood, there are many processes that occur dynamically, such as cell renewal and regeneration, so that the majority of the organisms are continuously developing.

The formation of a multicellular organism is the result of a global development that requires the integration of several processes. Indeed, it is essential that one single cell proliferate to give rise to many cells, which then differentiate in tissues and organs. Another type of differentiation is that which leads to the formation of the germ-line, which ensures the reproduction of an organism. Mechanisms of size control also occur to ensure that cells grow until a particular size and divide a certain number of times. The development is also subject to the pressure of evolution, which allows to select and to fix the advantageous changes in the developing species.

Therefore, the developmental biology is an integrative field of study that investigates the changes leading to the organism formation and to its correct functioning.

I2 *Drosophila melanogaster* as a model system

It is possible to compare the results obtained from studies in different model systems because the important biological pathways of the multicellular organisms are very conserved. Indeed, these organisms are formed by the use of general mechanisms with limited proteins and the morphological variety is achieved through the use of similar proteins, common to several pathways, used in different combinations and in distinct phases of the development. For this reason, the developmental biology uses model organisms that can be easily handled, with the aim of analyze the results and then extrapolate them to the whole animal kingdom.

One of the most studied model organisms is *Drosophila melanogaster*, also known as the fruit fly. Thomas Hunt Morgan was the first who studied this insect at the beginning of the twentieth-century (Morgan, 1910). Until now, it is the genetically best-known animal model. *Drosophila* has several characteristics that make it practical to study: it can be grown easily in a laboratory, it has only four chromosomes and a short life cycle. Furthermore, during the past decades, a lot of tools have been developed to genetically modify this insect. It is now possible to eliminate the expression of a specific gene by many tools, such as the RNA interference technique, and the use of deletions or mutagenesis. It is also possible to drive the

expression of a gene in a limited domain or in a precise developmental phase.

Thanks to the genome sequencing of several species we are now aware of the high conservation that exists between them. It has been estimated, for example, that the human species and *Drosophila* share approximately 60% of the genes (Adams, 2000); in addition, the same essential regulatory proteins control the principal biological pathways. For this reasons, the studies done on *Drosophila* have been used to extrapolate the results obtained to a general model of development.

I3 The life cycle of *Drosophila melanogaster*

Drosophila is a holometabolous insect, with a complete metamorphosis, as it reaches the adulthood from a larval stage.

Drosophila can live at a broad range of temperatures, between 17°C and 29°C. The length of its life cycle depends on the ambient temperature: it lasts 10 days at 25°C and 21 days at 17°C.

Figure I-1 represents the *Drosophila* life cycle: after fertilization, the adult female lays an egg (actually, it can lay about 100 eggs a day), in which the embryo develops until the formation of the first instar larva, that hatches 22 hours after fertilization. The larval phase lasts 4 or 5 days and during this time the larva grows considerably.

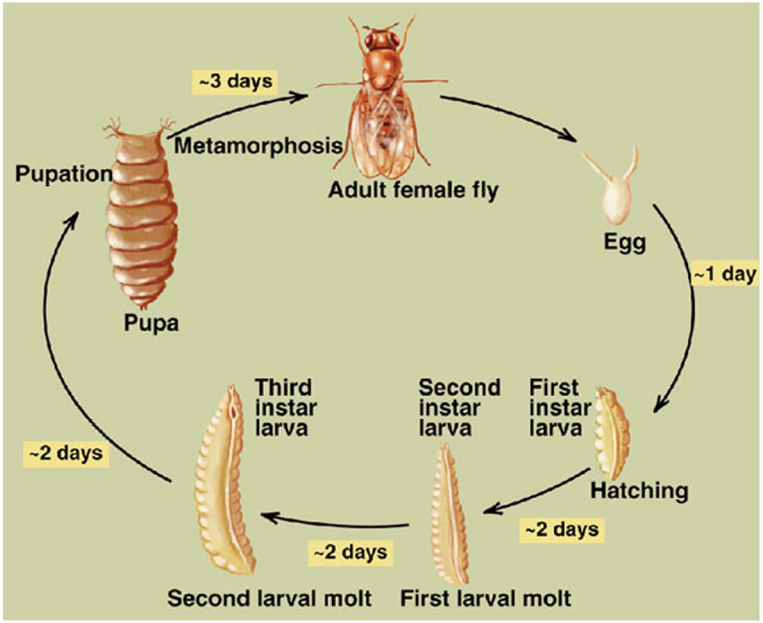


Figure I-1. The life cycle of *Drosophila melanogaster* at 25°C.
Taken from: http://www.zoology.ubc.ca/~bio463/lecture_13.html

The larva has two molting periods, during which the cuticle, mouth, hooks, and spiracles are shed. The larva is called an instar during the periods of growth before and after molting. Thus, the fruit fly has three instars. The *puparium* develops from the third instar, which becomes hard in texture and dark in color.

During the larval phase, the so-called imaginal discs are formed (Mandhavan, 1977). They are bag-shape epithelial structures that, during the pupal transformation, will form a great portion of the exoskeleton of the adult insect. The larva has two labial discs, two eye-antennal discs, six leg discs, two wing discs, two haltere discs and one genital disc (Fig. I-2). The metamorphosis from pupa to adult lasts 4 days and during this time the majority of the tissues are histolyzed, and the adult structures are formed from the imaginal discs. Once the metamorphosis is concluded, the adult fly get off the *puparium* and the life cycle starts again (Lawrence, 1992).

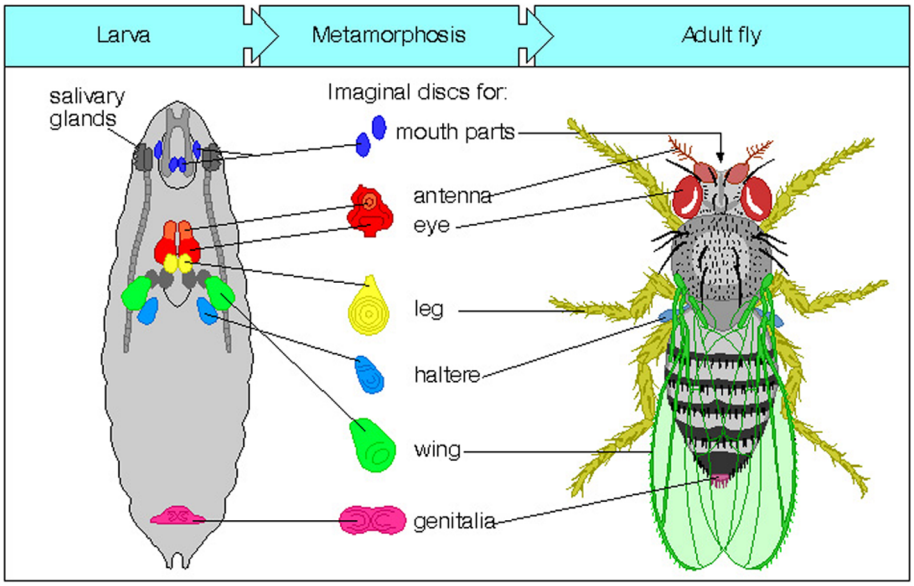


Figure I-2. The *Drosophila* imaginal discs. The imaginal discs of the *Drosophila* larva, that, after the metamorphosis, will give rise to the adult structures. Taken from: http://students.iitk.ac.in/projects/timeline

I4 The development of *Drosophila melanogaster*

I4A The embryonic development

The embryonic development begins after the fusion of the gamete nuclei, when a cycle of 10 fast divisions takes place in the middle of the embryo; these divisions are only nuclear, as the cell membranes are not formed, so they occur in a common cytoplasm, also known as *syncytium*. After the fast divisions, the nuclei reach the periphery of the embryo and the latter cellularizes to form a cellular blastoderm (cycle 14) (Fig. I-3). Each one of these divisions lasts 8-10 minutes and the cellular blastoderm is reached in 3 hours (Gilbert, 2003).

There is no transcription of zygotic genes until the cycle 13 and the RNAs and proteins necessary during de first hours of development are inherited from the mother (McKnight and Miller, 1976; Foe and Alberts, 1983).

Figure I-3. Early *Drosophila* embryonic development. The first nuclear divisions occur in the middle of the embryo (1-9), forming a *syncytium*. By the cycle 10 the nuclei migrate to the periphery and in the cycle 13 they cellularize, forming a cellular blastoderm (14) (Gilbert, 2003).

Once the nuclei reached the periphery, after the thirteenth division cycle, the plasma membrane of the oocyte invaginates between the nuclei, compartmentalizing each nucleus in one individual cell. This process generates a cellular blastoderm, in which all cells are located in a monolayer surrounding the egg cytoplasm (Fig. I-3) (Foe and Alberts, 1983).

During the following gastrulation, the posterior end of the embryo migrates up and over the dorsal surface in a process known as germ-band extension (Fig. I-4). Then the reverse process (called germ-band retraction) occurs, leaving a large open hole on the dorsal surface

of the embryo. Dorsal closure then takes place to close the hole by zipping up the surrounding epidermis (Leptin, 1995).

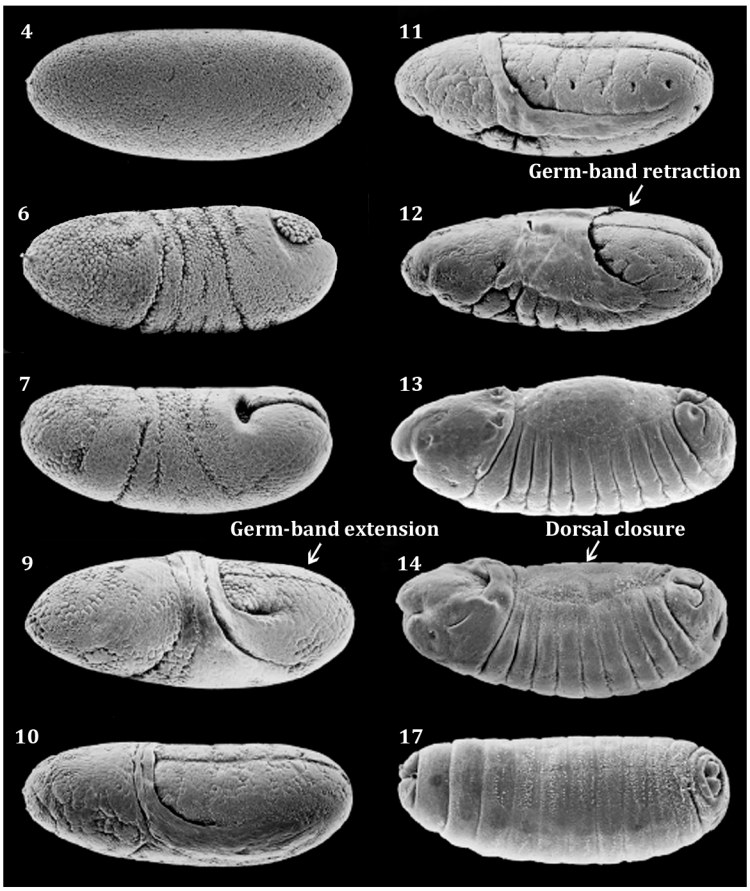


Figure I-4. *Drosophila* embryonic development. After the stage of cellular blastoderm, the gastrulation takes place, characterized by the extension and the following retraction of the germ-band. At the end of the gastrulation the embryo is fully segmented. The numbers indicate the developmental stages. Modified from: http://labs.fhcrc.org/parkhurst/embryo.html

I4Aa The anteroposterior axis

The anteroposterior polarity of the embryo and that of the following developmental stages originates in the egg. In the ovaries, the maternal genes are transcribed in mRNAs that localize to specific domains of the egg. These mRNAs encode regulatory proteins that distribute throughout the syncytial blastoderm, activating or repressing the expression of given zygotic genes in specific domains. Hereafter, the zygotic genes, regulated by the maternal proteins, start to be expressed in broad and partially overlapping patterns throughout the anteroposterior axis of the embryo. These genes are called *gap* and they are the first transcribed in the embryo (Fig. I-5). The different concentrations of the Gap proteins throughout the anteroposterior axis allow the transcription of the *pair-rule* genes, which divide the embryo into segments (Fig. I-5). The transcription of the distinct *pair-rule* genes gives rise to a pattern of 7 stripes, perpendicular to the anteroposterior axis. The Pair-rule proteins activate the transcription of the *segment polarity* genes, which subdivide the embryo into 14 segments (Fig. I-5). At the same time, the Gap, Pair-rule and Segment polarity proteins interact to regulate the *homeotic* genes, the transcription of which establishes the identity of each segment (shown below) (Carroll et al., 1988; Lawrence, 1992).

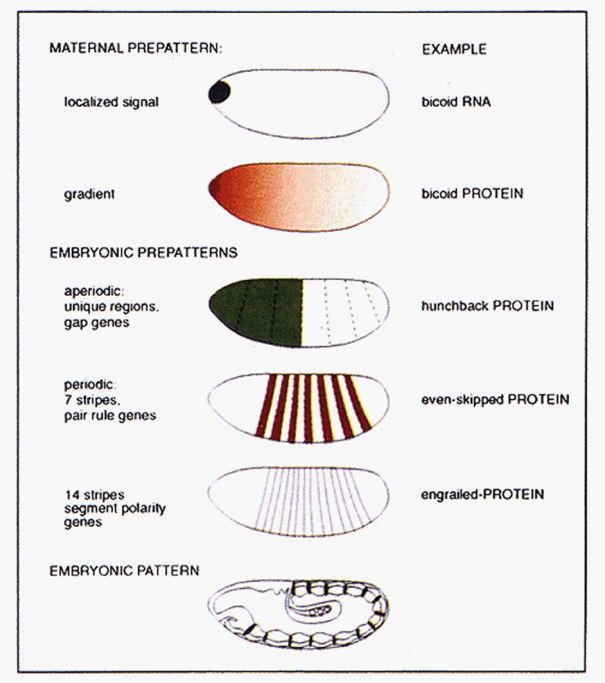


Figure I-5. The hierarchy of genes establishing the anteroposterior pattern in *Drosophia melanogaster.* The pattern is established through a genetic cascade of sequential activation that progressively delimits the embryo in segments, with unique identity, throughout the A/P axis. Taken from: http://gos.sbc.edu/n/nv/nvfig5.html

I4Ab The *homeotic* genes

The segmental identity is given by the *homeotic* genes (Lewis, 1978). Also known as *hox* genes, they lie in two complexes: the Antennapedia complex, which contains the genes that specify the head segments and part of the thoracic ones (*labial, proboscipedia, Deformed, Sex comb reduced, Antennapedia*) and the Bithorax complex (*Ultrabithorax, abdominal-A, Abdominal-B*), responsible for the segmental identity of the third thoracic segment and of the abdominal ones. The *hox* genes are conserved from flies to mammals, where they are subdivided in four clusters (Fig. I-6)(Lewis, 1978; Sanchez-Herrero et al., 1985; Duboule, 1992). Every *homeotic* gene is expressed in a specific domain and they show cross-regulatory interactions (Miller et al., 2001). Mutations in *hox* genes transform the fate of a segment into that of another one (McGinnis and Krumlauf, 1992).

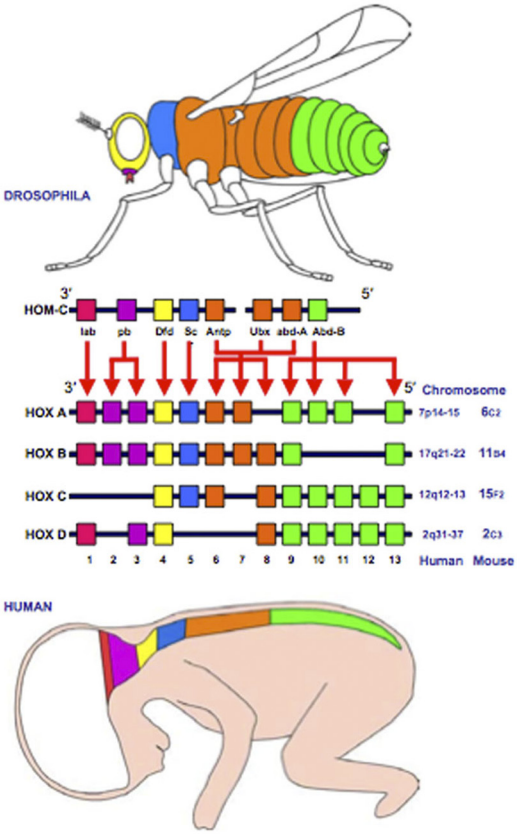


Figure I-6. Conservation between the *Drosophila* and mammals *hox* genes. The four *hox* gene clusters found in mammals are conserved from the *Drosophila* Hom-C complex in terms of nucleotide sequence and collinear expression. During embryonic development, the genes are expressed in a pattern that correlates with the chromosomal positioning, depicted here for human and mouse. The 3' genes are expressed both earlier and more anteriorly than the 5' genes (Lappin et al., 2006).

The *hox* genes are responsible for the segmental identity in *Drosophila* and vertebrates. In vertebrates they have been also shown to take part in other developmental processes like hematopoiesis, organogenesis and the development of the appendages (McGinnis and Krumlauf, 1992; Moens and Selleri, 2006).

The *homeotic* genes encode transcription factors that bind the DNA through the homeodomain. The latter is encoded by a sequence of 180bp known as homeobox. The homeodomain has a helix-loop-helix structure, typical of transcription factors that bind the DNA in the major groove of the double helix (Gehring and Hiromi, 1986).

Due to Hox proteins' functional specificity, one would expect that the different Hox proteins should bind to distinct DNA targets. However, *in vitro*, most Hox proteins bind to DNA with overlapping and low sequence specificities (Ekker et al., 1994; Mann, 1995). Indeed, the third helix of the Hox homeodomain binds the DNA at the conserved sequence TAAT (Otting et al., 1990). Therefore, the specificity of the DNA target sequence is low and *in vitro* experiments demonstrate that there is a weak affinity between the Hox proteins and the DNA (Hoey, 1998). The loose DNA recognition properties of the Hox proteins is difficult to understand, given the high degree of functional specificity they show *in vivo*.

It is currently known that the DNA binding affinity and specificity of the Hox proteins are increased through their interactions with cofactors.

The Hox cofactors had been identified as the genes that, when mutated, give rise to a homeotic phenotype similar to that of the *hox* mutants, without affecting Hox expression. Two of these Hox cofactors are *homothorax* (*hth*) and *extradenticle* (*exd*) (Peifer and Wieschaus, 1990; Rauskolb, 1993; Mann and Chan, 1996; Rieckhof et al., 1997), which were first discovered in *Drosophila melanogaster*.

I5 Homothorax and Extradenticle

Hth and Exd belong to the homeodomain-containing protein family known as TALE (Three Amino Acid Loop Extension, characterized by an extra three amino acids between helices 1 and 2), which includes highly conserved transcription factors (Bürglin, 1997; Rieckhof et al., 1997; Pai et al., 1998)

As shown in figure I-7, the TALE family is subdivided into two groups: the Prep/Meis family, including vertebrate Meis (Myeloid ecotropic insertion site) and Prep, fly Hth and worm Unc-62 (Uncoordinated-62), and the PBC family, including the vertebrate Pbx (pre-B-cell leukemia homeobox) proteins, fly Exd and worm Ceh-20 (*Caenorhabditis elegans* homeobox-20) (Burglin, 1994; Bürglin, 1997; Steelman et al., 1997; Burglin, 1998; reviewed by Moens and Selleri, 2006).

TALE homeobox genes were already present in the common ancestor of plants, fungi and animals and represent a branch distinct from the typical homeobox genes (Bürglin, 1997).

The Pbx1 and Meis1 proto-oncogenes code for divergent homeodomain proteins which are targets for oncogenic mutations in human and murine leukemias, respectively, and known to functionally interact with Hox proteins during embryonic development and/or oncogenesis (Moskow et al., 1995; Nakamura et al., 1996a; Nakamura et al., 1996b; Knoepfler and Kamps, 1997; Pineault et al., 2005; Sitwala et al., 2008).

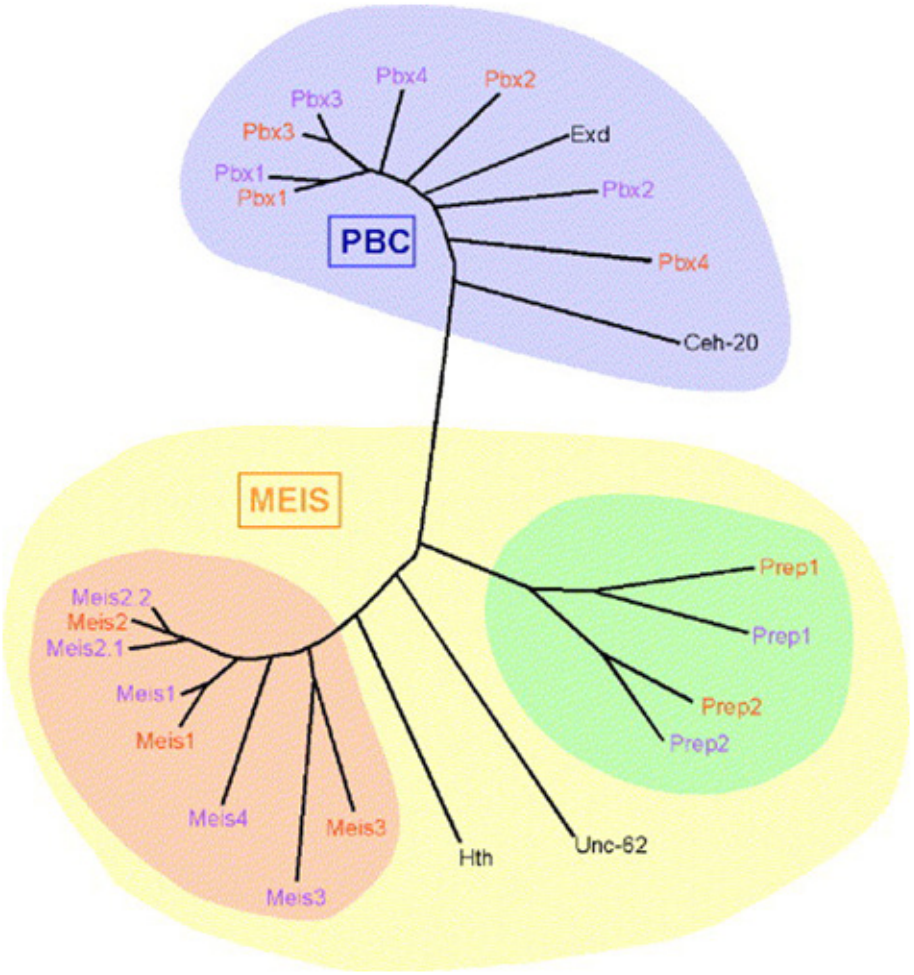


Figure I-7. Phylogeny of Hox cofactors. TALE homeodomain proteins are divided into two groups: the PBC family, including the vertebrate Pbx proteins, fly Exd and worm Ceh-20, and the MEIS family, including vertebrate Meis and Prep, fly Hth and worm Unc-62. Orange letters indicate mouse proteins, purple lettering indicates their zebrafish orthologs (Moens and Selleri, 2006).

I5A Protein structures

Two regions of *Drosophila* Hth are very similar to its relatives in vertebrates Meis and Prep (Moskow et al., 1995; Rieckhof et al., 1997; Steelman et al., 1997; Berthelsen et al., 1998b). One region in the N-terminal third of the protein, termed the Homothorax-MEIS (HM) domain, is the one used by Hth/Meis/Prep to bind Exd/Pbx (Rieckhof et al., 1997; Berthelsen et al., 1998b; Pai et al., 1998; Ryoo et al., 1999; Jaw et al., 2000). The HM includes two conserved sequences, known as HR1 and HR2 (Bürglin, 1997; Berthelsen et al., 1998b). A second region includes the homeodomain, whereby Hth/Meis/Prep binds the DNA (Fig. I-8) (Bürglin, 1997; Rieckhof et al., 1997; Kurant et al., 1998; Pai et al., 1998; Ryoo et al., 1999).

Exd shares three conserved domains with its vertebrate (Pbx) and *C. elegans* (Ceh-20) homologs (Burglin and Ruvkun, 1992; Rauskolb, 1993). Like Hth, Exd has an atypical homeodomain, characterized by an extra three amino acids between helices 1 and 2 (Burglin, 1994). The homeodomains of Hth and Exd present a high degree of homology (Rieckhof et al., 1997; Kurant et al., 1998; Pai et al., 1998). In addition to the homeodomain, two other domains (referred to as PBC-A and PBC-B) are conserved (Burglin and Ruvkun, 1992; Burglin, 1998). The first of them allows the binding between Hth/Meis/Prep and Exd/Pbx (Bürglin, 1997; Chang et al., 1997; Knoepfler and Kamps, 1997; Berthelsen et al., 1998a; Abu-Shaar et al., 1999) (Fig. I-8). The PBC-B domain includes a region corresponding to the predicted NES (nuclear export signal) of Exd and other functional motifs (Abu-Shaar et al., 1999; Calvo et al., 1999). Moreover, it has been shown to interact with the myosin II (Huang et al., 2003).

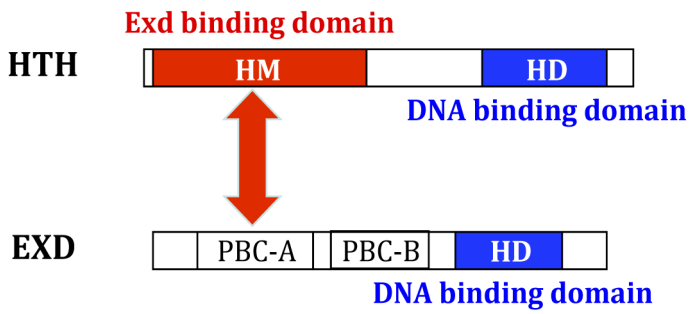


Figure I-8. Protein structures of Hth and Exd. Hth and Exd present a homeodomain (HD) by which they bind the DNA. Hth interacts with Exd through the HM domain (Hth) at the PBC domain (Exd). The latter presents two conserved regions called PBC-A and PBC-B and Exd binds Hth through the first one.

I5B The partners Hth and Exd

In *Drosophila,* Hth and Exd frequently work together and they are mutually dependent. Indeed Exd, that is expressed ubiquitously, is functional only when nuclear (Gonzalez-Crespo and Morata, 1995; Rauskolb et al., 1995; Aspland and White, 1997; Rieckhof et al., 1997; Pai et al., 1998) and Hth has been reported to be responsible for its translocation into the nucleus (Kurant et al., 1998; Abu-Shaar et al., 1999). Thus, Exd can be active solely in the presence

of Hth and the nuclear activity of Exd is indispensable to avoid Hth degradation (Abu-Shaar and Mann, 1998; Kurant et al., 1998; Pai et al., 1998). These two homeodomain proteins appear to require each other for their stable nuclear localization.

The current assumption is that, in the presence of Hth, both proteins associate and translocate into the nucleus to regulate downstream genes.

I5C The complex Hth/Exd and Hox proteins

The link between Hox and TALE proteins is their functional interaction, which was initially discovered in flies by genetic analysis (Mann and Affolter, 1998) and later supported by biochemical data (Moens and Selleri, 2006). Hth and Exd had been discovered for the first time in flies as Hox cofactors (Peifer and Wieschaus, 1990; Rieckhof et al., 1997; Ryoo et al.). Indeed, Hth binds Exd in the cytoplasm and translocates it into the nucleus, where they form a trimeric complex with a homeotic protein that, due to the three individual protein-DNA interactions, recognize Hox binding sites with higher affinity and specificity (indeed the DNA-binding affinity of the trimeric complex is higher than that of any of the proteins separately) (Fig. I-9) (Chan et al., 1994; Van Dijk and Murre, 1994; Ryoo and Mann, 1999). Exd directs the formation of the heterotrimeric complex by binding directly to both Hth and the Hox protein. In the absence of Exd and/or Hth, Hox proteins are unable to regulate many of their target genes (Rauskolb and Wieschaus, 1994; Ryoo and Mann, 1999; Ebner et al., 2005). Exd can bind to various domains in the anterior-Hox proteins, enabling complex formation and cooperative DNA binding (Passner et al., 1999; Piper et al., 1999; LaRonde-LeBlanc and Wolberger, 2003). There is no evidence of Hth binding to Hox proteins directly.

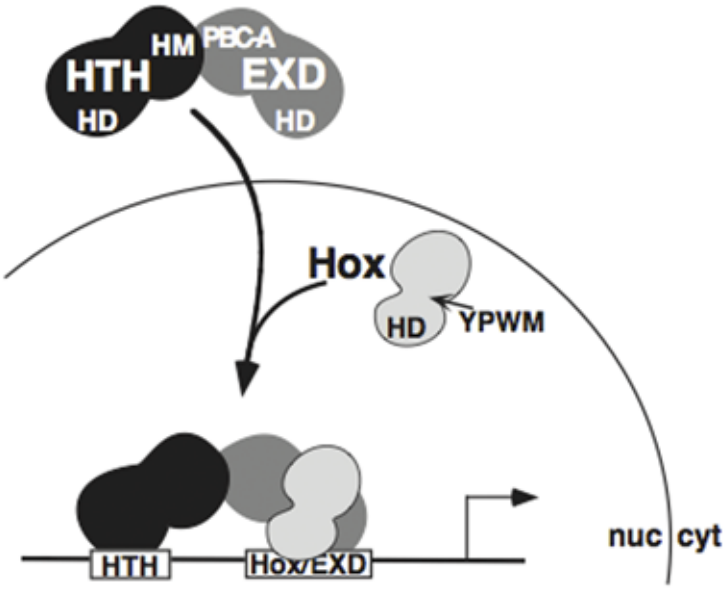


Figure I-9. Formation of the trimeric complex Hth/Exd/Hox on a Hox target enhancer. Hth binds Exd in the cytoplasm and translocates it into the nucleus.
Once in the nucleus, Hth remains associated with Exd and, together with a Hox protein, can assemble into a trimeric complex that requires an interaction between the Hox 'YPWM' motif and the EXD homeodomain. The Hox protein complex recognizes DNA via all three homeodomains (HD) with a high degree of sequence specificity. nuc, nucleus; cyt, cytoplasm (Ryoo et al., 1999).

I5D Functions of Hth/Exd

The homeodomain proteins Hth and Exd have multiple important functions during embryonic and adult development, not only in *Drosophila*, but also in vertebrates, suggesting a pivotal role during development. In addition to their function as Hox cofactors during embryonic development, they also play many Hox-independent roles. During larval development, *hth* is involved in the subdivision of wings and legs into proximal and distal domains (Abu-Shaar and Mann, 1998; Gonzalez-Crespo et al., 1998; Azpiazu and Morata, 2000; Casares and Mann, 2000; Azpiazu and Morata, 2002) and in the development of the posterior part of the notum or *scutellum* (Aldaz et al., 2005). Moreover, *hth* has been reported to be a selector gene in antennal development (Casares, 1998; Dong et al., 2002). The role of Hth/Meis proteins in limb development has been shown to be evolutionary conserved in fruit fly, chicken, and mouse (Mercader et al., 1999).

Hth has also been reported to be involved in eye development (Bessa et al., 2002) and photoreceptor cell-fate determination (Lappin et al., 2006), and it plays multiple roles in the formation of the adult fly organs, both as a selector of identity and an organizer of proximal-distal axis (Abu-Shaar and Mann, 1998; Casares, 1998; Wu and Cohen, 1999; Casares and Mann, 2000). Hth has been also reported to play a role in cell proliferation (Peng et al., 2009).

An unexpected role for *hth* in centric heterochromatin assembly in early embryos has been recently described (Salvany et al., 2009). This discovery points to an earlier and more basic role of *hth*, which makes it crucial for the correct assembly of chromatin structures, and for the proper division of the nuclei. This function seems to be conserved in vertebrates as the homologue of *hth*, *prep1*, is required to maintain genomic stability (Iotti et al., 2011) and this role could be related to the implications of these transcription factors in several cancers and acute diseases. Indeed, *meis1* had been found mutated in distinct types of leukemias (Moskow et al., 1995; Thorsteinsdottir et al., 2001; Yeoh et al., 2002; Hisa et al., 2004; Zeisig et al., 2004; Wang et al., 2006; Caslini et al., 2007; Rosales-Avina et al., 2011), in ovarian cancer (Gilbert, 2003), in neuroblastoma (Spieker et al., 2001; Dirk Geerts, 2003) and in restless legs syndrome (RLS) (Winkelmann et al., 2007; Xiong et al., 2009).

I6 The complexity of the *hth locus*

The *hth locus* spans over a genomic region bigger than 100 kilobases on the right arm of the third chromosome. The *hth* pre-mRNA includes 14 short exons separated by very long introns; the exons 2-6 encode for the conserved HM domain and the HD is encoded by the exons 11-13 (Fig. I-10A).

The *hth* pre-mRNA is subject to alternative splicing, by which 6 mature mRNAs are transcribed (Flybase). Three of them, the HthRA, HthRC and HthRH, include both the HM and HD

domains and, due to their high similarity, they had been reported as full-length Hth isoforms (FLHth) (Noro et al., 2006). The remaining transcripts are the short ones as they have only the HM domain (HthRE and HthRF) or exclusively the HD domain (HthRG) (Fig. I-10B.)

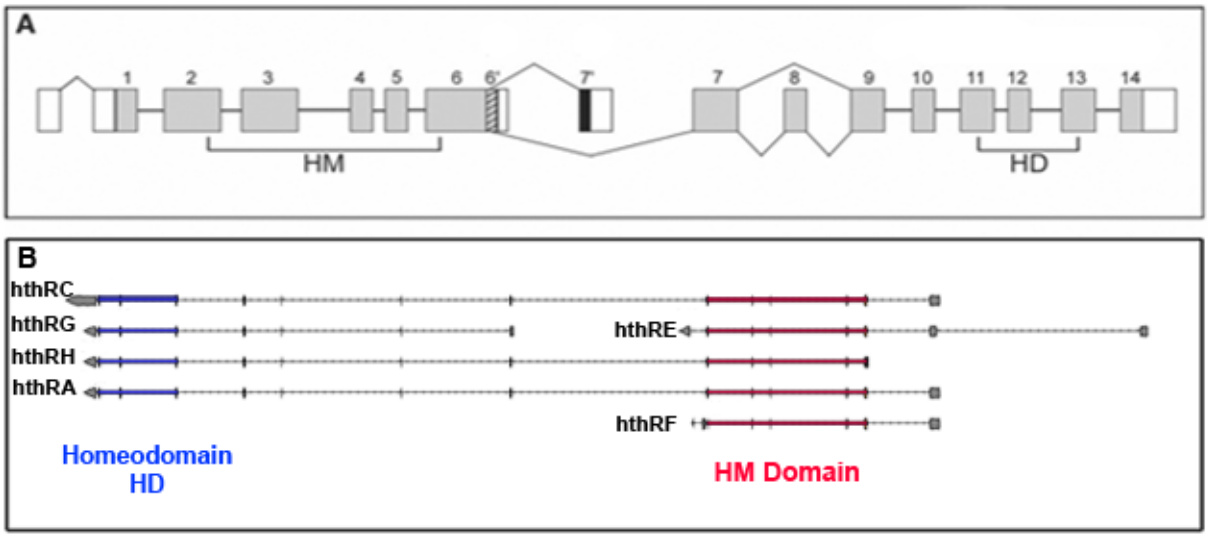


Figure I-10. The alternative splicing of *hth* and the resulting transcripts. (A) Genomic organization of *hth locus* with the 5' end on the left (not shown to scale). White and gray boxes denote noncoding and protein-coding exons, respectively, while angled lines represent sites of alternative splicing. Modified from Noro et al., 2006 **(B)** The *hth locus* spans over 100 Kb and gives rise to 6 different mRNAs, coding for distinct isoforms: the full-length isoforms HthRA, HthRC and HthRH, containing the HD and the HM domains, two short isoforms, containing only the HM domain (HthRE and HthRF) and one short isoform HthRG, which has only the homeodomain. The 5' end is on the right. Modified from Flybase.

The genomic structure of the *hth/meis* genes also displays a high conservation between species, especially in the similarity of their long introns, suggesting the presence of regulatory elements in these noncoding sequences (Irimia et al., 2011). The alternative splicing of the TALE proteins does not occur exclusively in flies, as mammalians present three Meis proteins and splice variants of Meis1 and Meis2 that encode HDless isoforms have been identified (Yang et al., 2000). Furthermore, it has been reported that these different mammalian isoforms are not expressed in the same tissues and that they are not functionally redundant (Crist et al., 2011).

All this information points to different roles of the different Hth/Meis isoforms during development. A first attempt to study them in *Drosophila* was made by Noro and colleagues in 2006. However, they only consider two of the isoforms (the Flhth and one HM containing one) and use a mutant that was not well characterized. Therefore, the conclusions of their study were incorrect.

With my work I would like to analyze in detail the expression and the role of each Hth isoform, in order to understand their functional diversity during development.

II. OBJECTIVES

OBJECTIVES

1. To analyze the expression and the distribution of the Hth isoforms during *Drosophila* embryonic development and in *Drosophila* cell lines.

2. Try to separate the functions of the distinct isoforms during *Drosophila* embryonic development.

3. To study the regulatory interactions between the different Hth isoforms.

4. To study in more detail the relationship between Hth and Exd.

III. MATERIALS AND METHODS

MATERIALS AND METHODS

M1 *Drosophila* strains

I used the *yw Drosophila* strain as the wild-type (wt) strain. The Df(3R)hth mutant strain from Exelisis (6158) has been generated by the Bloomington Drosophila Stock Center. It eliminates almost all *hth* transcripts (the hthRG remains in the deficiency). The PM1 mutant strain has been generated in our laboratory (by S. Aldaz): the recombination of the P elements PBac{WH}f04473 and P{XP}hth[d06865] led to the deletion of the *hth* genomic region lying between them (Parks et al., 2004); in this mutant only the hthRE and the hthRF isoforms can be transcribed.

I used the following transgenes and Gal4 lines: salGal4 (Cruz et al., 2009), babGal4 (Azpiazu and Morata, 2002), nulloGal4 (Kunwar et al., 2003), UbxGal4 (Herranz and Morata, 2001; de Navas et al., 2006), UASFlhth (Aldaz et al., 2005), UAShthRE, UAShthRF, UAShthRG. I used the Df(3R)hth to generate the Dfhth;nulloGal4 strain and the DfhthUbxGal4 recombinant.

M2 Real time RT-PCR

I collected embryos overnight, extracted their RNA using the GE Healthcare extraction kit. I retrotranscribed total RNA obtained from each type of embryo with the Super Script III First-Strand Synthesis SuperMix for ABI (PN 11752250) qRT-PCR. The real time PCR was performed with a BioRad CFX 384.

To compare the amplification obtained for each couple of primers, I used standard samples of each amplicon to estimate the efficiency of the primers. This procedure allowed me to do an absolute quantification of the distinct *hth* mRNAs.

The primers used to amplify the Flhth and hthRG isoforms were:
Flfw: 5'-AATGCAGACGCTTCCCGGCG-3'
Flrw: 5'-CCATGGCGTCGTGGCCATAT-3'
To amplify the hthRE isoform, I used the following primers:
REfw: 5'-CTCTCCGCACATGACGCACG-3'
RErw: 5'-AAAAGTCGGGCCGGACTAAAA-3'
The primers used to amplify the hthRF isoform were:
RFfw: 5'-GTCCTATATTGGCGAGCTACAATG-3'
RFrw: 5'-CAACTTCTTCTGCGTTCGGATTTG-3'
To amplify the hthRG isoform, I used the following primers:
RGfw: 5'-GGCCACGACAATCTTGAACATTTG-3'
RGrw: 5'-GGTGATGCGTTAGCTGATTGTT-3'

M3 *In situ* hybridization with immunostaining of embryos and probes labeling

I cloned specific cDNAs of the HD-containing *hth* isoforms, the hthRE, the hthRF and the hthRG isoforms in a pGEM-T vector. I then synthesized *hth* antisense RNAs for each cDNA and labeled them with digoxigenin (Roche). I collected embryos overnight, dechorionated them for 2 minutes in commercial bleach and immediately fixed them in a mixture of 5% formaldehyde and heptane for 20 minutes at room temperature. I removed the aqueous phase and added methanol. I removed the vitelline membranes by vigorous shaking and I washed the embryos in methanol several times. I then rehydrated the embryos by washing them with a mixture of PBT (PBS with 0,1% tween) methanol 1:1 and then with PBT. I subsequently fixed again the embryos in 4% paraformaldehyde in PBT and washed them in PBT. I then incubated the embryos for at least 1 hour at 55°C with the hybridization solution (HS). I denatured the probe at 80°C for 5 minutes and hybridized the embryos overnight at 55°C. I washed the embryos once with HS at 55°C, once with PBT-HS 1:1 at 55°C and 4 times at room temperature with PBT. I then incubated the embryos with an anti-digoxigenin antibody for 1 hour, washed them four times in PBT and incubated for 1 hour with a biotinylated antibody. After four washes in PBT, I incubated the embryos for 20 minutes with the AB complex, washed three times and incubated for 20 minutes with tyramide in the darkness. I performed the further steps in the darkness. After several washes with PBT, I incubated the embryos overnight with the primary antibody at 4°C. After three washes in PBT, I incubated the embryos with the proper secondary antibody for 1 hour. I then washed the embryos three times in PBT and mounted them in Vectashield (Vector Laboratories). I took the images in a confocal laser MicroRadiance microscope (Leica) and subsequently processed them using Adobe Photoshop. The HS consists of 50% formamide, 5 x SSC (1 x SSC is 150mM NaCl, 15 mM sodium-citrate), 50mg/ml heparin, 0.1% Tween20 and 40mg/ml tRNA. The antibodies used were: anti-DIG 1:200 (sheep) (Roche), biotinylated 1:200 (sheep) (Jackson ImmunoResearch Laboratories), AB complex 1:100 (Vector Laboratories), tyramide 1:50 (PerkinElmer), anti-Hth 1:500 (Azpiazu and Morata, 2002) (rabbit), Alexa 555 1:200 (rabbit) (Invitrogen) and Topro3 1:1000 (Invitrogen). All the washes I did, lasted 20 minutes.

M4 Western blot of embryos and cell lines

I collected each type of embryo overnight, dechorionated them in commercial bleach for 2 minutes and homogenized them in lysis buffer with proteases inhibitors (Roche) at 4°C. For the cells, I directly homogenized them in lysis buffer at 4°C. I quantified the protein concentration by Bradford assay. I ran 50 μg of each sample in a SDS-PAGE gel (12% acrylamide or a gradient of 8%-16% acrylamide) and transferred it to nitrocellulose membranes (Amersham Biosciences). I incubated the membranes in blocking buffer for 30 minutes and incubated

them with the primary antibody diluted in the blocking buffer overnight at 4°C. I washed four times the membranes with TBS-T. For the fluorescent blots revelations, I incubated the membranes with the proper fluorescent secondary antibody (Odissey) for 1 hour in the darkness. I performed the further steps in the darkness. I washed the membranes four times with TBS-T and visualized the proteins using the Odissey Infrared Imaging System. For the visible blots, I incubated the membrane with a biotinylated antibody for 1 hour, then washed four times with TBS-T. Finally, I incubated the membrane with the AB complex for 20 minutes, washed 4 times and incubated with a staining solution for 1 minute in the darkness. I exposed the membranes to X-ray film (Agfa) for 5 minutes. All the washes lasted 5 minutes. The lysis buffer consists of 50 mM Tris pH 8.0, 150 µM NaCl, 1% triton X-100, 0,5% sodium deoxycholate and 1 µM ethylenediaminetetraacetic acid. The TBS-T is made of 20 mM Tris-HCl pH 8.0, 150 mM NaCl and 0,2% Tween-20. The blocking buffer contains 3% (w/v) nonfat dry milk and 1% (w/v) bovine serum albumine fraction V (BSA, Sigma) in TBS-T. The staining solution is made of 50 µl luminol 250 mM, 25 µl coumaric acid 90 mM, 3 µl hydrogen peroxide in 10 ml of Tris 100 mM pH 8,5. The primary antibodies used were: anti-Hth 1:1000 (rabbit) (Azpiazu and Morata, 2002), anti-HthCterminal 1:250 (goat) (Santa Cruz Biotechnology), anti-HthRE 1:500 (guinea pig), anti-HthRF 1:500 (rabbit). The respective preimmune serums were used at the same concentration. The secondary antibodies used were: IRDye 800 1:10000 (rabbit) (Odissey), IRdye 800 1:10000 (guinea pig) (Odissey), IRDye 800 1:10000 (goat) (Odissey), biotinylated 1:1000 (goat) (Invitrogen), AB complex 1:100 (Vector Laboratories).

The following antibodies were generated by Biomedal using specific peptides:

anti-HthRE: PSTQDFSPLEETYASYRIKHEADF

anti-HthRF: CVSTPFAGAHGPILASYNAVVHPCS

M5 Cell culture and immunostaining of S2 and ML-DmD8 cell lines

I grew S2 and DmD8 cell lines as a suspension culture at 25°C in flasks to a density of $1x10^7$ cells/ml. The culture medium of the S2 cells was the Insect X-Press (Lonza), supplemented with 7% of heat-inactivated FBS (fetal bovine serum), penicillin G (50 units) and streptomycin sulfate (50 µg/ml). The medium of the DmD-8 cells was the Shields and Sang M3 insect medium (Sigma), supplemented with 10% of heat-inactivated FCS (fetal calf serum), insulin (10 µg/ml) and BPYE (2,5 g/l of bactopeptone and 1 g/l of yeast extract). For the immunostaining experiments, I let the cells adhere on a polylisine slide (Sigma) for 30 minutes and then fixed them in 4% paraformaldehyde for 5 minutes. I washed the cells twice with PBS and blocked them with a solution containing PBT (PBS+0,1% tween) and 3 % BSA (Sigma) for 10 minutes. I then incubated the cells with the primary antibody diluted in blocking solution for 1 hour, washed them three times with PBS and incubated for 1 hour with the proper secondary antibody in the darkness.

After 2 washes in PBS and 1 in distilled water, I added Vectashield (Vector Laboratories) to the slide. I took the images in a confocal laser MicroRadiance microscope (Leica) and subsequently processed them using Adobe Photoshop. The antibodies used were: anti-Hth 1:100 (rabbit) (Azpiazu and Morata, 2002), anti-HthCterminal 1:100 (goat) (Santa Cruz Biotechnology), anti-HthRE 1:100 (guinea pig), anti-HthRF 1:100 (rabbit), Alexa 555 1:200 (goat) (Invitrogen), Alexa 488 1:200 (rabbit) (Invitrogen), Alexa 555 1:200 (guinea pig) (Invitrogen), anti-Mannosidase II 1:100 (rabbit) (Millipore), Topro3 1:1000 (Invitrogen), Phalloidin-Alexa 647 1:200 (Invitrogen).

M6 PCRs

To obtain the genomic DNA of wt and PM1 mutant embryos and of the parental strain PBac{WH}f04473, I collected them, homogenized in extraction buffer, and incubated in the same buffer at 70°C for 30 minutes. I extracted the DNA with phenol/chloroform and precipitated it with potassium acetate and isopropanol. To obtain the cDNA of wt and PM1 mutant embryos, I collected them overnight and extracted their total RNA using the GE Healthcare extraction kit. I retrotranscribed the RNA obtained from the embryos with the First Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche). The PCR reactions were performed with the Expand High Fidelity PCR System (Roche), using the GenAmp PCR System 9700 (Applied Biosystems).

I used the following primers:

PCR n.1: fw: 5'-TTCGCGCTATTTAGAAAGAGAGAGC-3'
rw: 5'-TAAGCATTGGTAAGCTGGATTCTGG-3'

PCR n.2 and n.5: fw: 5'-AGGAATTTCCACGGCAAATGG-3'
rw: 5'-GTAACGCTAATCACTCCGAAC-3'

PCR n.3: fw: 5'-CGCACTTATTGCAAGCATACG-3'
rw: 5'-AACAATTCTGTTGGCGGCTGG-3'

PCR n.4: fw: 5'-TTTTTCGAGGCTCAGACCCC-3'
rw: 5'-CAGCCTTCCACTGCGAATCA-3'

PCR n.6: fw: 5'-CTTTGTGTTGTTGTTATTGTTGTG-3'
rw: 5'-ACCTGCCCAATTAAAATGTAACGT-3'

PCR n.7: fw: 5'-CCCAGGACTATAGCTCTCTAAA-3'
rw: 5'-TGCCTCGCTTTTCTCGCATTTC-3'

PCR n.8: fw: 5'-TCTATACACCGCATCCAGGT-3'
rw: 5'-GGTTTGTTGTTGCTGGTGCT-3'

PCR n.9: fw: 5'-CTCTCCTCCCCCAATTGTAT-3'
rw: 5'-GGCTATAATAAACCGCTGGGAATCGGTTCG-3'

PCR n.10: fw: 5'-TATTGGCGAGGTACAATGCGG-3'

rw: 5'-GGGTGTTCAGTGTGGGTTAAG-3'
PCR n.11: fw: 5'-GGCCACGACAATCTTGAACATTTG-3'
rw: 5'-GGTGATGCGTTAGCTGATTGTT-3'
PCR n.12: fw: 5'-AATGCAGACGCTTCCCGGCG-3'
rw: 5'-CCATGGCGTCGTGGCCATAT-3'
PCR n.13: fw: 5'-TCGACGAACGGGACACCACC-3'
rw: 5'-GGTTTGTTGTTGCTGGTGCT-3'

The extraction buffer contains Tris 0,1M pH 9.0 (Sigma), ethylenediaminetetraacetic acid 0,1 M (Merck), sodium dodecyl sulfate 1% (Merck).

M7 Generation of transgenic flies

I used the Gal4/UAS system (Brand and Perrimon, 1993) to induce ectopic expression of the Hth isoforms. To do so, I cloned three *hth* cDNAs (hthRE, hthRF and hthRG) in the pUASt vector, and injected them in *yw* embryos.

M8 Preparation of larval cuticles

I collected embryos overnight and then aged them additional 12 hours. I dechorionated first instar larvae in commercial bleach for 3 minutes and then removed the vitelline membranes using heptane-methanol 1:1. After washing with methanol and 0.1% Triton X-100, I mounted the larvae in Hoyer's lactic acid (1:1) and allowed them to clear at 65°C for at least 24 hours. I took the images in a Zeiss Axiophot optic microscope and subsequently processed them using Adobe Photoshop.

M9 Immunostaining of embryos and discs

I collected embryos overnight, dechorionated and immediately fixed them in a mixture of 5% formaldehyde and heptane for 20 minutes at room temperature. I removed the aqueous phase and added methanol. I removed the vitelline membranes by vigorous shaking and I washed the embryos in methanol several times. I then rehydrated them and blocked in 10% BSA (Sigma). I did the incubation with the primary antibody overnight in PBT (PBS, 0.1% Tween).

I dissected the discs in PBS and fixed them in 4% paraformaldehyde for 20 minutes at

room temperature. I then washed them in PBS, blocked in blocking buffer (PBS, 0.3% Triton, 1% BSA) and incubated overnight with the primary antibody diluted in blocking buffer at 4°C. I washed embryos and discs in PBT, and I added the appropriate fluorescent secondary antibody for 1 hour at room temperature in the darkness. After further washes in PBT in the darkness, I mounted embryos and discs in Vectashield (Vector Laboratories). I took the images in a confocal laser MicroRadiance microscope (Leica) and subsequently processed them using Adobe Photoshop. The antibodies used were: anti-Hth 1:500 (rabbit) (Azpiazu and Morata, 2002), anti-Exd 1:200 (rat) (Gonzalez-Crespo and Morata, 1995), anti-HthCterminal 1:100 (goat) (Santa Cruz Biotechnology), anti-HthRE 1:200 (guinea pig), anti-HthRF 1:200 (rabbit).

M10 *In situ* hybridization of discs and probe labeling

I cloned *exd* cDNA in a pBluescript vector, synthesized *exd* antisense RNA and labeled it with digoxigenin (Roche). I dissected the discs in PBS and fixed them in 4% paraformaldehyde (in PBS) for 20 minutes at room temperature. I subsequently washed the discs in PBS, fixed again in 4% paraformaldehyde (in PBT) and washed in PBT. I then washed the discs in increasing percentages of hybridization solution (HS), kept at -70°C at least 72h and prehybridized them for 1h at 55°C. I denatured the probe at 80°C for 10 minutes and hybridized the discs overnight at 55°C. I subsequently washed the discs in HS at 55°C, in PBT at room temperature and then incubated them 2h with anti-DIG antibody (Roche) 1:2000. I then washed the discs 3 times in PBT, once in staining buffer (SB) and incubated them with a solution containing NBT (nitro blue tetrazolium) and BCIP (5-bromo-4-chloro-3-indolyl phosphate). I stopped the reaction by washing with PBT and I mounted the discs in glycerol. The HS consists of 50% formamide, 5 x SSC (1 x SSC is 150mM NaCl, 15 mM sodium-citrate), 50mg/ml heparin, 0.1% Tween20 and 40mg/ml tRNA. The SB consists of 4M NaCl, 1M $MgCl_2$, 1M Tris pH 9,5 and 10% Tween20.

IV. RESULTS

RESULTS

R1 Hth isoforms in wild type embryos and cell lines

R1A *hth* mRNAs in wild type embryos

R1Aa Relative amounts of the different *hth* isoforms in wild type embryos

The first approach I have taken to study the different *hth* isoforms was to measure their relative amounts in the embryo. I have performed qRT-PCRs for the different *hth* mRNAs using specific primers, when allowed. Due to their high similarity, it was impossible to distinguish between the 3 long isoforms, which I have grouped together and named as full-length isoforms (Flhth). Other than this, I have designed specific primers for the hthRG isoform, as well as for the hthRF and hthRE ones (Fig. R-1A). Note that the primers used to amplify Flhth isoforms also recognize the hthRG isoform. This means that the levels obtained for the Flhth isoforms actually correspond to the sum of Flhth and hthRG isoforms (all the HD-containing isoforms).

As can be observed in the figure R-1B, the more represented mRNAs are the HD-containing isoforms, which are 8 times more abundant in the embryos than the hthRF. The amount of hthRG, although detectable, is approximately 125 times less abundant than the Flhth isoforms and 16 times less represented than the hthRF. With this technique I was unable to detect the transcript of the hthRE isoform.

R1Ab Distribution of the *hth* RNAs in wild type embryos

I then wanted to analyze the spatial and temporal distribution of the different isoforms. The low general levels of all the isoforms make them hard to be detected by the traditional *in situ* hybridization technique. With specific probes against the different isoforms, I was only able to detect the cytoplasmic HD-containing ones (Fig. R-2A). The probes designed to be specific for the short isoforms hybridize to an exon region in the isoform I wanted to visualize and an intron in the rest of the isoforms. The *in situ* hybridization using this probe gave a strong signal in nuclear speckles (Fig. R-2B, R-2C and R-2D), suggesting that it was recognizing the immature long RNAs being processed in the nucleus. This strong signal made it difficult to distinguish the milder signal in the cytoplasm that corresponds to the mature short isoforms.

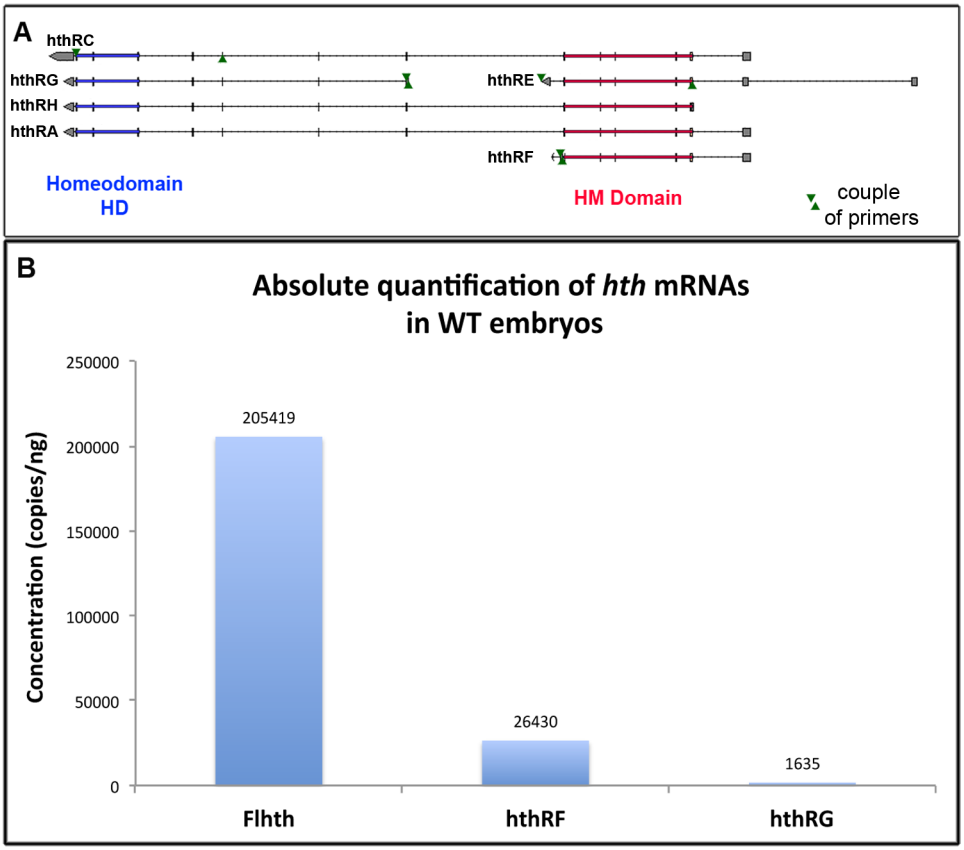


Figure R-1. *hth* transcripts. (A) The *hth locus* spans over 100 Kb and gives rise to 6 different mRNAs, coding for distinct isoforms: the full-length isoforms HthRA, HthRC and HthRH, containing the HD and the HM domains, two short isoforms, containing only the HM domain (HthRE and HthRF) and one short isoform HthRG, which has only the homeodomain. The 5' end is on the right. Modified from Flybase. **(B)** Absolute quantification of *hth* mRNAs in wild type embryos. The primers used to amplify the *hth* mRNAs are shown in A (green triangles). The full-length *hth* mRNAs (Flhth) are the most abundant ones, the hthRF mRNA is about 8 times less abundant than the Flhth mRNAs. There is a very low amount of hthRG mRNA and the quantity of the hthRE mRNAs is not detectable with this technique.

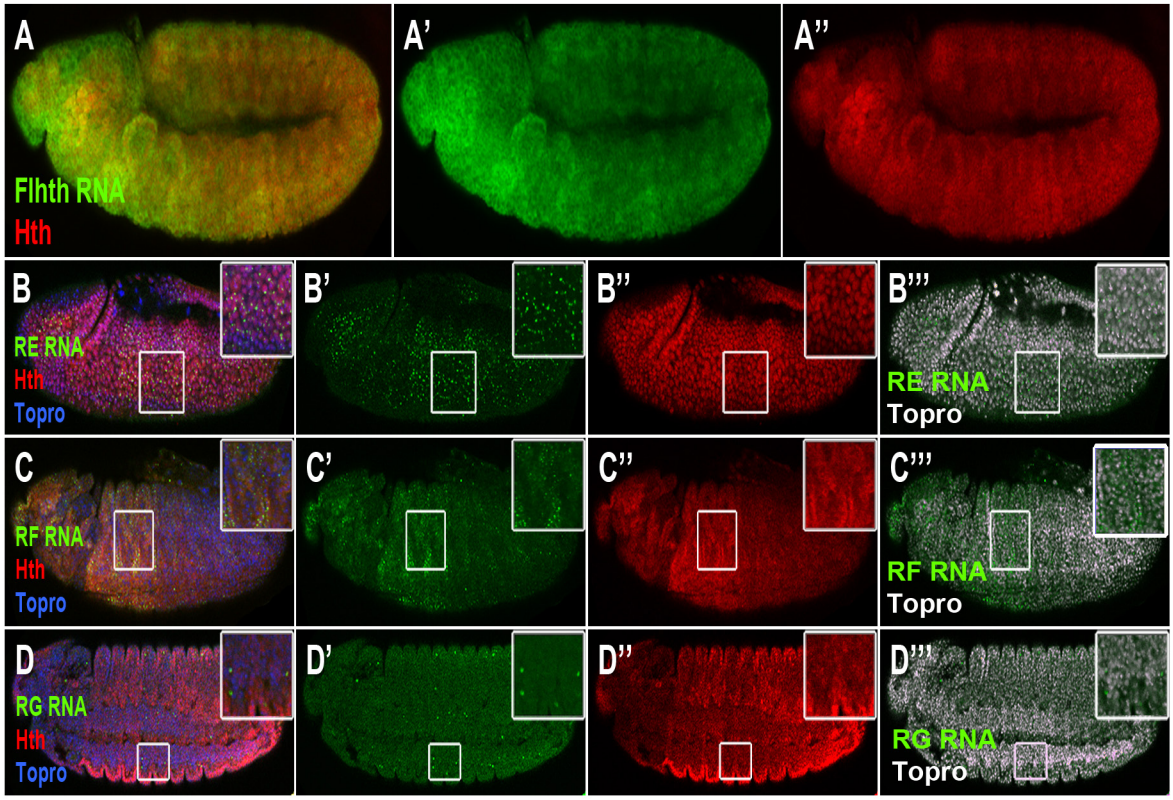


Figure R-2. Distribution of *hth* mRNAs in wild type embryos. (A) The mature HD-containing mRNAs (green) are ubiquitous and cytoplasmic in the embryo and the Hth protein (red) is mostly nuclear. **(B)** The hthRE RNAs (green) are accumulated in nuclear speckles, indicating that they are the immature long RNAs being processed; the Hth protein is ubiquitous and nuclear (red). **(C)** The mature hthRF mRNAs are cytoplasmic and the signals coming from the nuclear speckles indicate the long RNAs being processed; the expression of the Hth protein is shown in red. **(D)** The low amounts of the mature hthRG mRNAs (green) are distributed in the cytoplasm and some expression of the long immature RNAs in the nuclear speckles is visible; the expression of the Hth protein is shown in red.

R1B Expression of Hth isoforms in wild type embryos

R1Ba *In vivo*

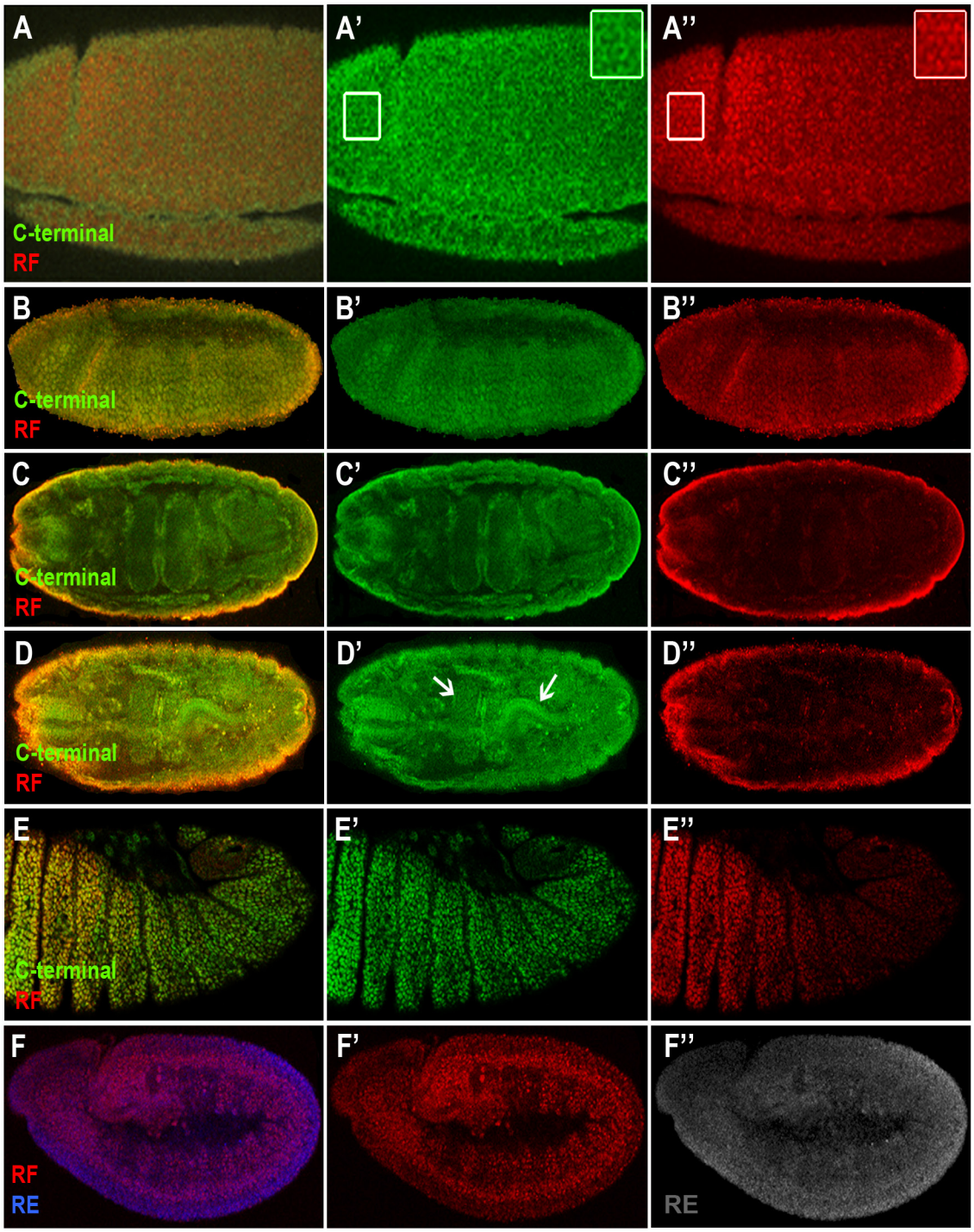
The next approach I decided to take was to analyze the expression of the different protein isoforms. To do so I used, when possible, specific antibodies for the distinct isoforms. There is a commercial available antibody that recognizes the C-terminal part of Hth, which corresponds to FlHth and HthRG (HD-containing isoforms). In our laboratory, two antibodies against the HthRE and HthRF isoforms were generated, using specific peptides of the different proteins (see materials and methods). I tried all three antibodies in wild type embryos.

I detected differences in the distribution of the HD-containing isoforms and the short HthRF. As shown in figure R-3A, the short HthRF isoform starts to be detected in the nuclei of the early blastodermic embryo (stage 5). In contrast to this, I do not observe any nuclear expression of the HD-containing isoforms until short after germ-band extension. Up this moment, the expression of the isoforms is very similar (Fig. R-3B). However, the distribution of FlHth is almost uniform in the nuclei along the anteroposterior axis, whereas HthRF shows higher levels or nuclear expression in the anterior segments and lower nuclear distribution towards the posterior (Fig. R-3E).

There are also some differences in the levels of expression in the internal organs of the embryo (Fig. R-3C) and, looking in more detail, I could also detect other more subtle differences between the HD-containing isoforms and HthRF in some internal organs of the embryo (Fig. R-3D).

The HthRE isoform shows a general distribution in wild type embryos, which is very faint and hard to be detected with our specific antibody (Fig. R-3F). The difficulties that I encounter trying to visualize the protein in wild type embryos could be due to the general low levels of the transcript, undetectable by the qPCR experiment. However, I believe that this expression is true because I am not able to detect such a distribution with the pre-immune serum.

Figure R-3. Analysis of the distribution of Hth isoforms during embryonic development with specific antibodies. (A) In the early blastoderm embryo (stage 5), the HthRF (red) is nuclear and ubiquitous, whereas the HD-containing isoforms (green) are still cytoplasmic. **(B)** During germ-band extension, the HD-containing isoforms (green) become nuclear and have an expression similar to HthRF (red). **(C)** In the interior of the embryo, the HD-containing isoforms (green) and HthRF (red) show differences in the levels of expression. **(D)** There are subtle differences of expression between the HD-containing isoforms (green) and HthRF (red) in the interior of the embryo (arrows). **(E)** The HD-containing isoforms (green) are expressed ubiquitously, whereas the HthRF (red) shows higher levels of expression in the anterior part of the embryo. **(F)** The HthRE (blue) shows a ubiquitous and very low expression during embryonic development.



R1Bb Western Blot

I decided to further analyze the Hth isoforms by western blot analysis. To do so, I extracted the total amount of proteins present in wild type embryos of all stages and ran a polyacrylamide gel, which I then transferred to a membrane that was hybridized with a general Hth antibody, and with the three Hth specific antibodies listed above. To be sure of the specificity of the bands that I detected in the blots, I compared each of them with the pattern of bands obtained with their preimmune serum (Fig. R-5).

The predicted molecular weights of the Hth isoforms are as follow: the three FlHth between 49,6 kDa and 52,8 kDa, the short HthRE and HthRF approximately 28 kDa and the HthRG 23 kDa (Flybase). However, it should also be taken into account that several post-translational modifications are predicted for all the Hth isoforms (http://prosite.expasy.org/); they include myristoylations, phosphorylations and amidations (reported in Fig. R-4) that could alter their predicted size.

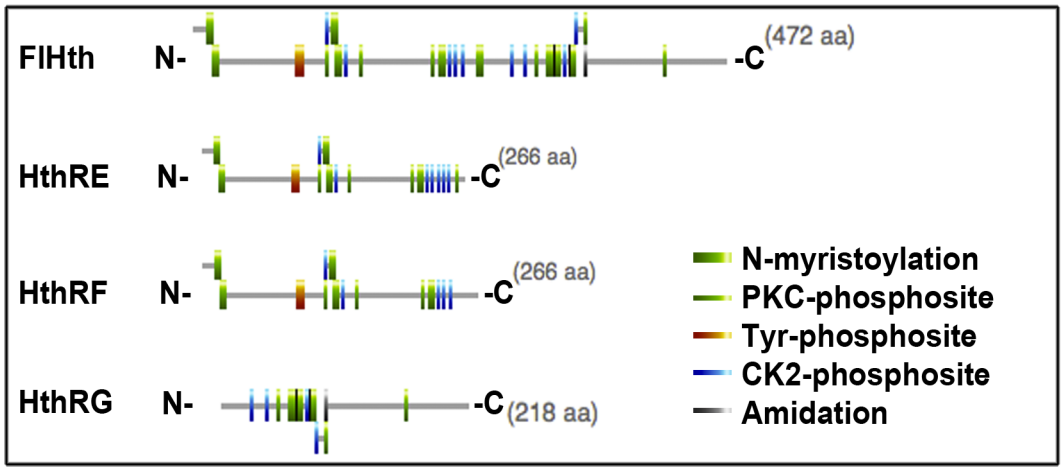


Figure R-4. Predicted post-translational modifications of Hth isoforms. Several post-translational modifications are predicted for all the Hth isoforms (http://prosite.expasy.org/).

Using the general Hth antibody, that should recognize all the isoforms, I could detect at least four specific bands (Fig. R-5). One of them is just below the 36 kDa band and could correspond to HthRF, as I am able to detect the same band using the specific antibody against the HthRF (see below and Fig. R-8). I also detected a couple of bands between 50 kDa and 64 kDa, certainly corresponding to the FlHth isoforms. I assume that the molecular weights that I observe are slightly higher that the predicted ones, because the isoforms are post-translationally modified. The highest band than I detect in the blot lies over the 98 kDa band, and it could correspond to the maternal Hth isoform, as it does not disappear in the blots done with the zygotic *hth* mutant embryos (see below and Fig. R-17A). The remaining bands are not specific, as I am able to detect them also with the pre-immune serum.

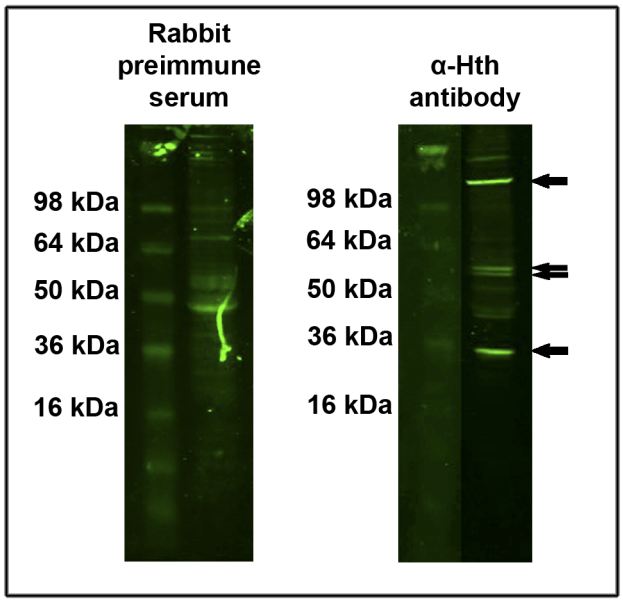


Figure R-5. Hth isoforms present in wild type embryos. The blot revealed with the general Hth antibody shows four specific bands: one just below 36 kDa corresponding to the HthRF, a couple of bands between 50 kDa and 63 kDa matching with the FlHth isoforms, and the last one over 98 kDa corresponding to the maternal isoform.

Using the antibody raised against the C-terminal part of Hth, that recognizes the HD-containing isoforms, two specific bands are visible (Fig. R-6). One of them is just below 36 kDa and the other one lies at 64 kDa. These weights are slightly higher than the expected ones for the FlHth isoforms and the HthRG, and therefore I think that these isoforms are also post-translationally modified.

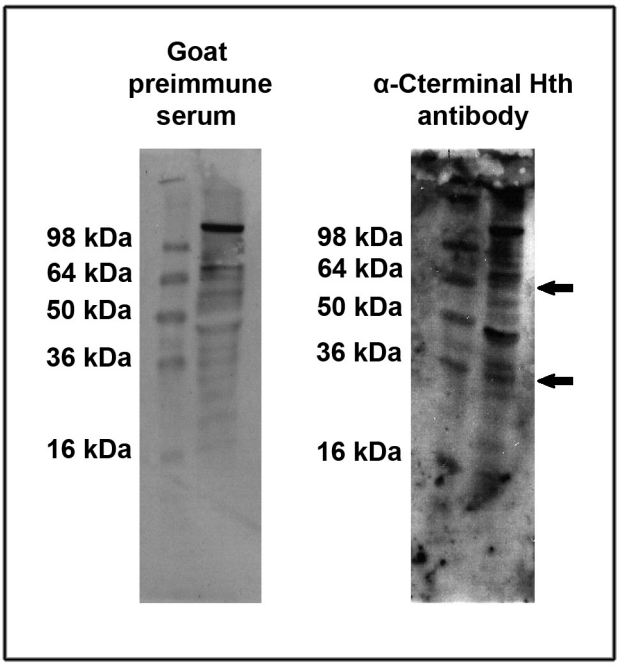


Figure R-6. HD-containing Hth isoforms present in wild type embryos. The blot revealed with the antibody raised against the C-terminal part of Hth shows two specific bands: one just below 36 kDa and another one at 64 kDa. They correspond to the post-translationally modified HthRG and FlHth isoforms, respectively.

When using the specific antibody against HthRE, I could detect a band a bit over 50 kDa and another one over 64 kDa (Fig. R-7). This observation also points to post-translational modifications of the HthRE isoform. The remaining bands are not specific, as they can be detected with the pre-immune serum.

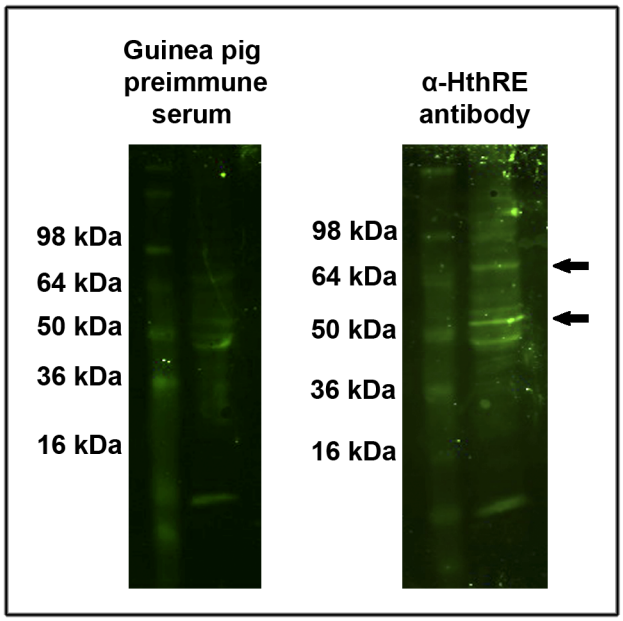


Figure R-7. HthRE isoform in wild type embryos. The blot revealed with the specific antibody against HthRE shows two specific bands: one slightly higher than 50 kDa and another one over 64 kDa, indicating the presence of post-translational modifications of the HthRE isoform.

As can be observed in figure R-8, the blot hybridized with the specific antibody against HthRF shows three specific bands: one just below 36 kDa, another one slightly lower than 64 kDa and the highest one at 98 kDa. As all these weights are higher than the predicted for the HthRF isoform, I assume that they reproduce the result of post-translational modifications of the protein. The remaining bands are not specific.

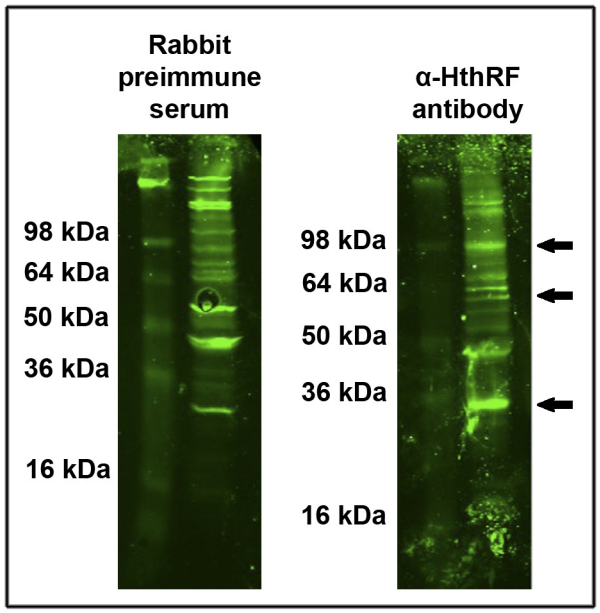


Figure R-8. HthRF isoform in wild type embryos. The blot revealed with the specific antibody against HthRF shows three specific bands: one just below 36 kDa, another one slightly lower than 64 kDa and the highest one at 98 kDa, reproducing the result of post-translational modifications of the protein.

R1C Expression of Hth isoforms in Schneider 2 and ML-DmD8 cell lines

R1Ca *In vivo*

I wanted to analyze the distribution of the Hth isoforms in two different *Drosophila* cell lines: the Schneider 2 cells, also known as S2, and the ML-DmD8 cell line. The S2 cell line was derived from a primary culture of late stage (20-24 hours old) embryos (Schneider, 1972), in which Hth is known to be expressed. The ML-DmD8 is a cell line obtained from the wing disc of the third instar larval stage (Ui et al., 1987), which expresses high levels of Hth (reported in the website https://dgrc.cgb.indiana.edu/cells/tiling-search.html).

When I looked at the distribution of the distinct Hth isoforms in the S2 cells, I noticed that they have different expression patterns. As can be observed in figure R-9A, the HD-containing Hth isoforms (visualized by the antibody against the C-terminal part of Hth) are predominantly in the cytoplasm and only slightly expressed in the nucleus where they accumulate in a few points, usually colocalizing with the HthRF isoform. The latter is more ubiquitous, as it is expressed both in the nucleus and in the cytoplasm of these cells, and sometimes it is only cytoplasmic. Moreover, the HthRF isoform shows a doted distribution in the cytoplasm and on the DNA, colocalizing occasionally with the HD-containing Hth isoforms.

The HthRE isoform exhibits a characteristic expression in S2 cells (Fig. R-9B), as it strongly accumulates in the cytoplasm, more often surrounding the nucleus, and it is expressed at low levels in the remaining cell body.

The distribution of the Hth isoforms in the ML-DmD8 cell line is different. The figure R-9C shows the expression of the HD-containing Hth isoforms in these cells; as can be observed, these isoforms frequently accumulate in the nucleus, with a low expression in the cytoplasm.

In some of the ML-DmD8 cells the HthRF isoform is cytoplasmic, having there a heterogeneous distribution, and in other cases it also accumulates in the nucleus. As in the S2 cells, even in the ML-DmD8, the HthRE isoform aggregates in the cytoplasm strongly and heterogeneously, in some cases around the nucleus (Fig. R-9D).

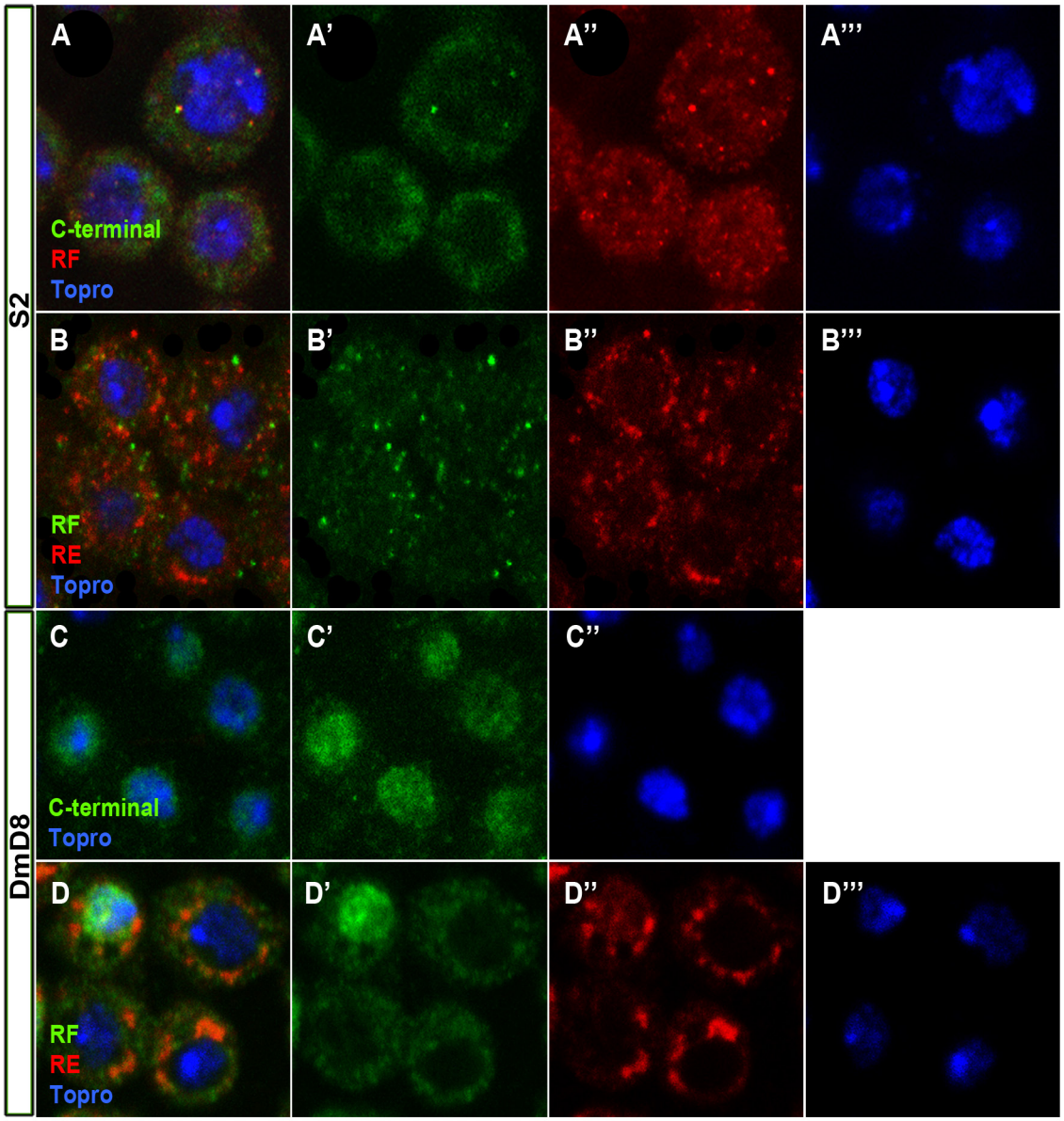


Figure R-9. Expression of Hth isoforms in S2 and DmD8 cell lines. (A) In S2 cells the HD-containing isoforms (green) and the HthRF isoform (red) are mostly cytoplasmic and sometimes co-localize in points on the DNA. **(B)** In S2 cells the expression of the HthRF isoform (green) is not uniform in the cytoplasm and the HthRE isoform (red) strongly accumulates in the cytoplasm, more often surrounding the nucleus. **(C)** In DmD8 cells the HD-containing isoforms (green) frequently accumulate in the nucleus, with a low expression in the cytoplasm. **(D)** In DmD8 cells the HthRF isoform (green) is heterogeneously distributed in the cytoplasm and in some cases it accumulates in the nucleus; the HthRE isoform (red) aggregates in the cytoplasm strongly and heterogeneously, in some cases around the nucleus.

R1Cb Expression of the HthRE isoform in the Golgi apparatus

Given the characteristic expression of the HthRE isoform in S2 and ML-DmD8 cell lines, I hypothesized that this isoform could be accumulating in the Golgi apparatus. To verify this, I did double stainings with Mannosidase II, which is a Golgi marker, and the anti-HthRE antibody.

I observed a partial colocalization of the HthRE isoform and the Golgi apparatus in S2 and ML-DmD8 cell lines (Fig. R-10A and R-10B). As the Golgi apparatus is integral in modifying, sorting and packaging proteins for exocytosis or use within the cell, a possible explanation for the observed colocalization is that the HthRE isoform is secreted. In support of this hypothesis is the fact that I often observe the HthRE isoform located in the surface of the S2 cells, or right outside the cell membranes (Fig. R-10C).

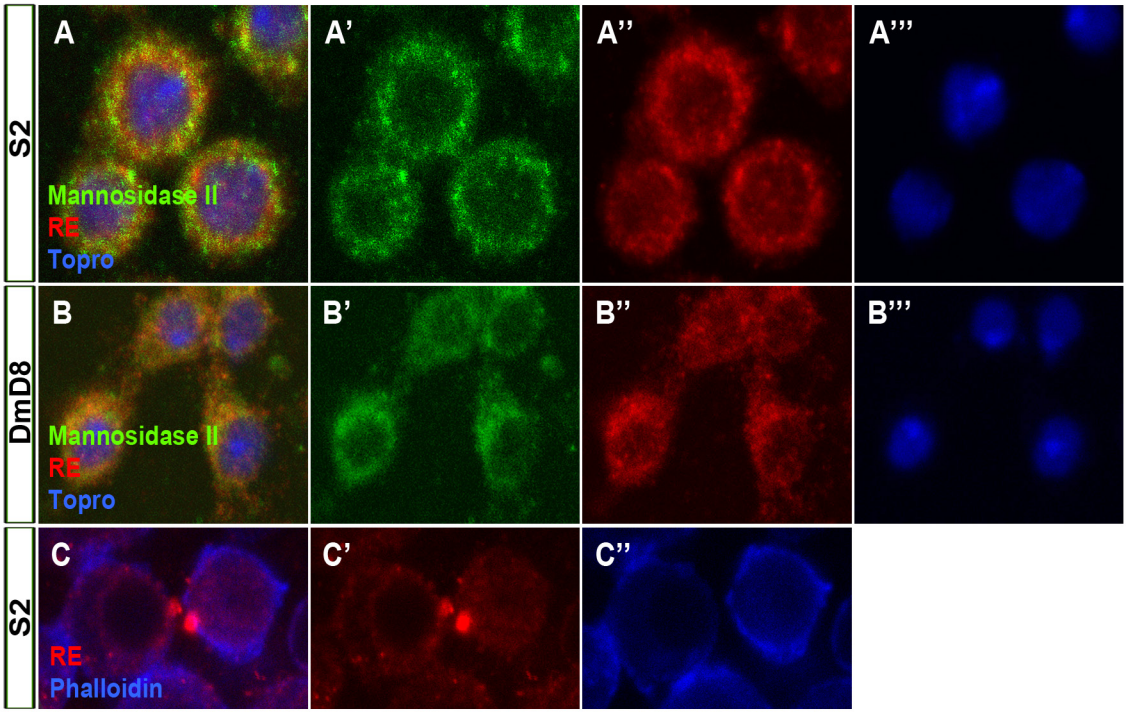


Figure R-10. Expression of the HthRE isoform in the Golgi apparatus and on cell membranes. **(A)** Colocalization of the HthRE (red) and the Golgi marker Mannosidase II (green) in S2 cells. **(B)** Colocalization of the HthRE isoform (red) and the Golgi marker Mannosidase II (green) in DmD8 cells. **(C)** Localization of the HthRE isoform (red) on the cell membranes of S2 cells, visualized with anti-Phalloidin (blue).

R1Cc Western blot

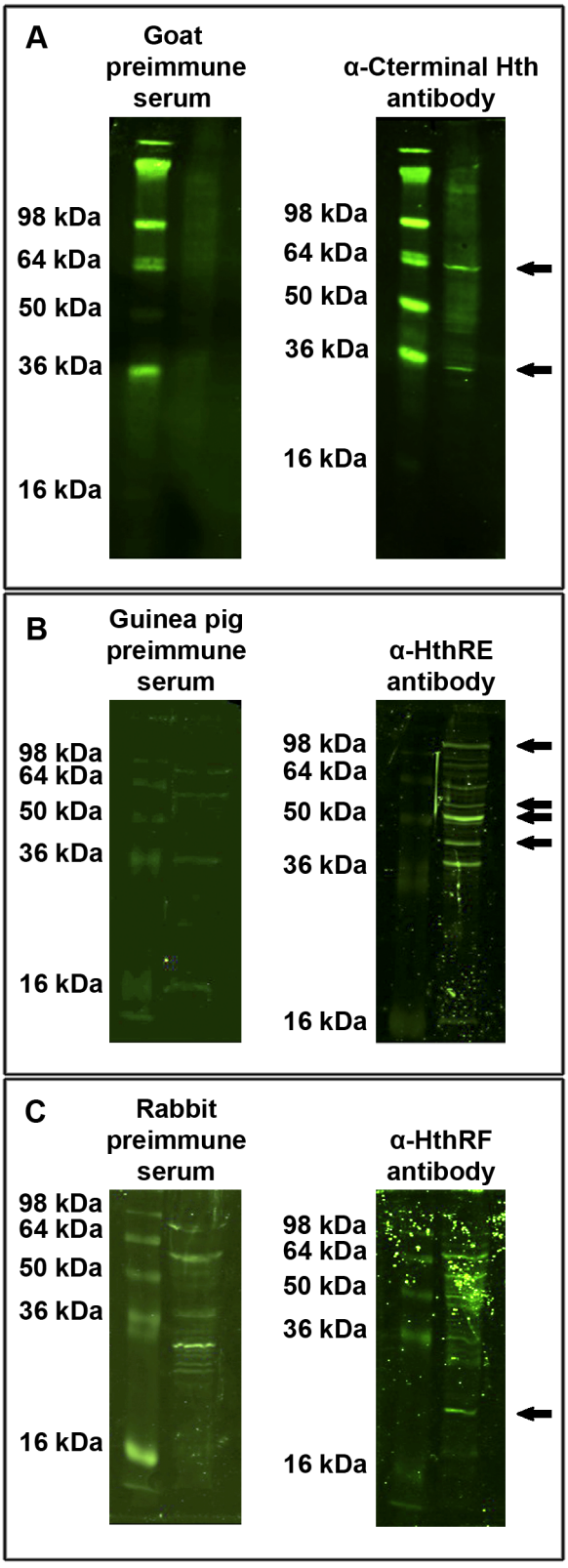
I then tried to visualize the Hth isoforms present in S2 cells by western blot analysis. To do so, I extracted the total amount of proteins present in these cells and hybridized the blots with the antibodies against the C-terminal part of Hth, the HthRE and HthRF isoforms. To assure the specificity of the bands that I saw in the blots, I compared each of them with the pattern of bands obtained with their preimmune serum.

Using the antibody against the C-terminal part of Hth, that recognizes the HD-containing isoforms, I saw two specific bands (Fig. R-11A). One of them is just below 36 kDa and the other one lies at 64 kDa. These weights are a bit higher than the ones predicted for the HD-containing isoforms, therefore I think that in the S2 cells these isoforms are post-translationally modified.

When I used the specific antibody against the HthRE, I detected in the blot several specific bands (Fig. R-11B): between 36 kDa and 50 kDa, at 50 kDa, between 50 kDa and 64 kDa and one slightly higher than 98 kDa. Again, I think that these weights could reflect post-translational modifications of the HthRE isoform. The remaining bands are not specific.

As can be observed in figure R-11C, the blot hybridized with the specific antibody against HthRF shows one specific band between 16 kDa and 36 kDa, corresponding to 28 kDa, the predicted molecular weight for this isoform. The remaining bands are not specific. This observation suggests that in S2 cells, in contrast to what happens in embryos, the HthRF isoform is not post-translationally modified.

Figure R-11. Hth isoforms in S2 cell line. (A) HD-containing isoforms in S2 cells. The blot revealed with the specific antibody against the C-terminal part of Hth shows two specific bands: one is just below 36 kDa and the other one lies at 64 kDa, reproducing the result of post-translational modifications of the proteins. **(B)** HthRE isoform in S2 cells. The blot revealed with the specific antibody against the HthRE isoform shows four specific bands: between 36 kDa and 50 kDa, between 50 kDa and 64 kDa and one slightly higher than 98 kDa, reproducing the result of post-translational modifications of the protein. **(C)** HthRF isoform in S2 cells. The blot revealed with the specific antibody against the HthRF isoform shows one specific band between 16 kDa and 36 kDa.


A
Goat preimmune serum
α-Cterminal Hth antibody
98 kDa
64 kDa
50 kDa
36 kDa
16 kDa
98 kDa
64 kDa
50 kDa
36 kDa
16 kDa
B
Guinea pig preimmune serum
α-HthRE antibody
98 kDa
64 kDa
50 kDa
36 kDa
16 kDa
98 kDa
64 kDa
50 kDa
36 kDa
16 kDa
C
Rabbit preimmune serum
α-HthRF antibody
98 kDa
64 kDa
50 kDa
36 kDa
16 kDa
98 kDa
64 kDa
50 kDa
36 kDa
16 kDa

R2 The *hth* mutants: Dfhth and PM1

R2A Description of the mutants Dfhth and PM1

I decided to study the different functions that the distinct isoforms might have during embryonic development. To do so, I used two deficiencies generated within the *hth* genomic region, which I called Dfhth and PM1. The Dfhth mutant is an Exelisis deficiency generated by the Bloomington Drosophila Stock Center; the PM1 mutant has been previously generated in our laboratory (by S. Aldaz).

As shown in figure R-12A, the Dfhth embryos lack the 5´genomic region of *hth*, and in these, only the hthRG isoform can be transcribed. The PM1 mutant is a deficiency within the *hth locus*, that was obtained through the recombination of two P elements inserted in the *hth* genomic region (Parks et al., 2004): the PBac{WH}f04473 located at the 3' end of the *locus* and the P{XP}hth[d06865] situated between the last exon of the hthRE and the first exon of the hthRG. Recombination between these two P elements caused the loss of the 3' genomic region. Therefore, in the PM1 mutant only the hthRE and hthRF isoforms can be transcribed (Fig. R-12B).

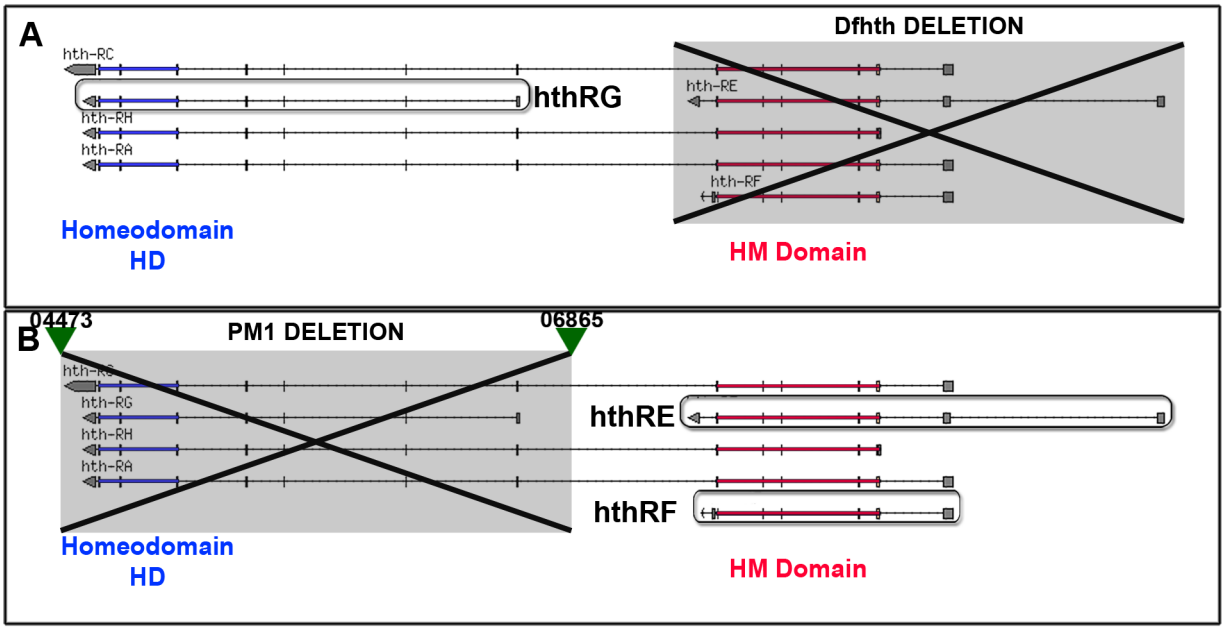


Figure R-12. Schematic representation of the deletions in the *hth locus* present in the *hth* mutants. **(A)** The mutants Dfhth lack the 5' genomic region, and in these, only the hthRG isoform can be transcribed. **(B)** The PM1 mutants present a deficiency in the 3' genomic region, caused by the recombination of the P elements 04473 and 06865 (green triangles). In these mutants only the hthRE and hthRF can be transcribed.

R2Aa Molecular characterization of the PM1 mutant

I wanted to check that the deficiency of the PM1 was properly generated, in other words, that the recombination took place where it should occur and that the genomic region in between the two P-elements was deleted.

To this purpose I generated primers that annealed in the genomic region outside the deficiency, in the P elements, and in the genomic region supposedly deleted. Figure R-13A shows the four couples of primers used to confirm the presence of the deletion (the ones corresponding to the PCRs 1-4). As expected, I obtained amplification when using the primers of the P element located at the 3' end and the region outside the deficiency (PCR n.1), but not with the primers of the genomic region deleted in the deficiency (PCR n.2) (Fig. R-13B). I performed the negative PCR n.2 (the one inside the deficiency) with the parental fly strain PBac{WH}f04473 as a control, and, as expected, I was able to amplify this genomic region (PCR n.5 in Fig. R-13B).

For the 5' region, I did also not obtain amplification with the primers annealing inside the presumably deleted region (PCR n.3), whereas the PCR with the primers of the genomic region outside the deletion was positive (PCR n.4) (Fig. R-13B). Unfortunately I was unable to do the control PCR with the P-element located at the 5' end (the P{XP}hth[d06865]) because the parental stock got lost after generating the deficiency. For this reason, I decided to use primers that anneal inside the deleted genomic region. With these I was able to confirm that the recombination between the P elements did not occur properly, as the 3' region of the gene, supposed to be deleted by the recombination event, was still present in the mutants. Indeed, I was not able to amplify the intron between exons 8 and 9 (PCR n.6), but the last intron (PCR n.7) and the last exon (PCR n.8) were still present in homozygous embryos (Fig. R-13C).

Because the genomic nature of the deficiency was not completely clear, I decided to extract the total RNA of homozygous mutant embryos and performed RT-PCRs to check out which RNAs were actually transcribed. As expected, both hthRE and hthRF were present in the mutants (PCR n.9 and PCR n.10 respectively in Fig. R-13D) and the first exon of the hthRG was absent (PCR n.11 in Fig. R-13D). In addition to this, I was also able to detect a RNA that contains exons 9, 10, 11, 12, 13 and 14 (PCR n.12 in Fig. R-13D).

As in the PM1 mutants a short aberrant RNA was being transcribed from the 3' end of the *hth locus*, I wanted to check the possibility that an abnormal full-length RNA, which could has all the exons, except for the 7th and 8th, was also generated. To verify the presence of this aberrant transcript, I performed a PCR using primers that anneal in the sixth exon and in the last one. I did not obtain amplification in this case (PCR n.13 in Fig. R-13D), suggesting that in the PM1 mutants only three short RNAs were actually transcribed: the normal hthRE and hthRF, and an anomalous RNA holding the last six exons.

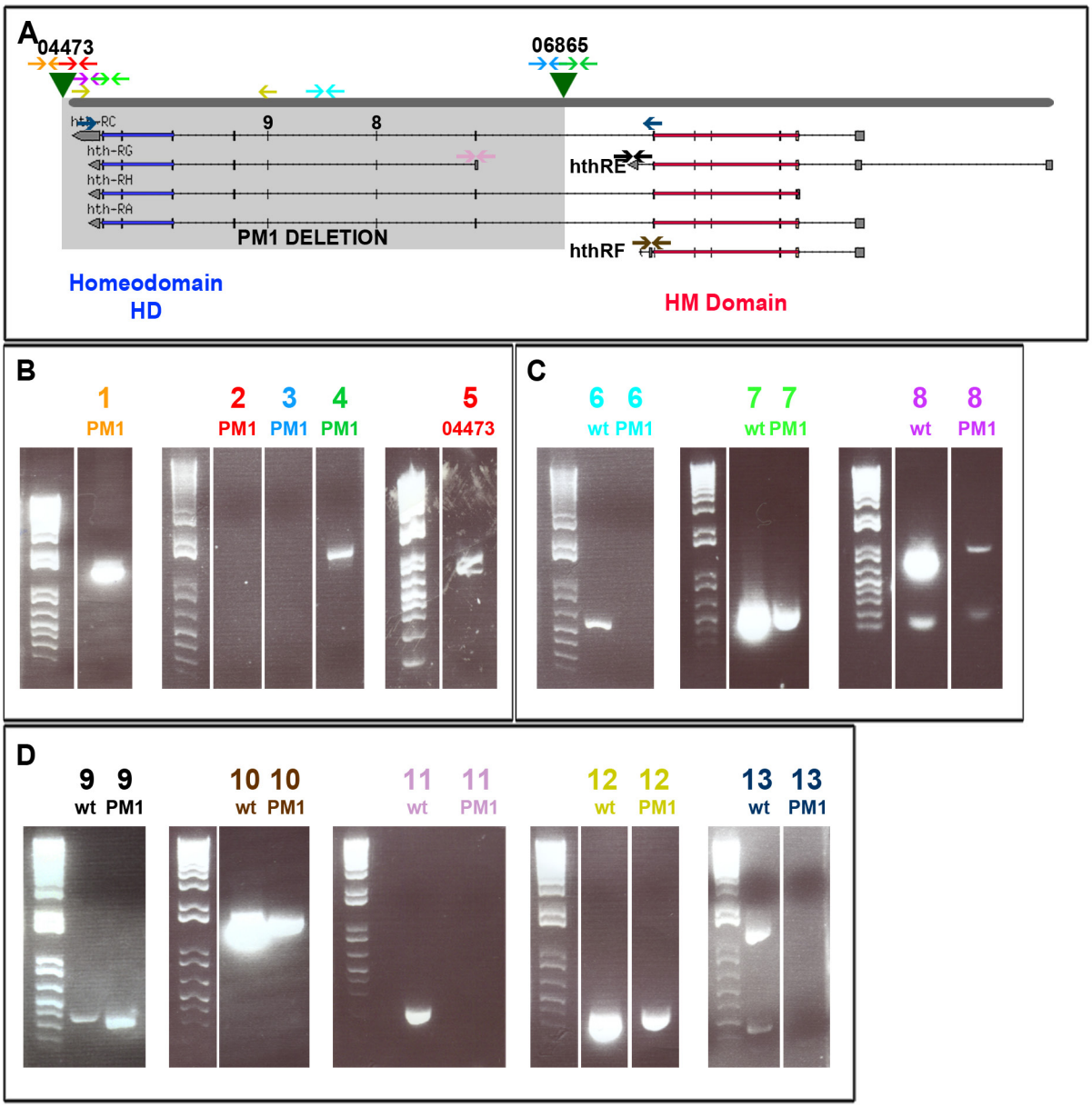
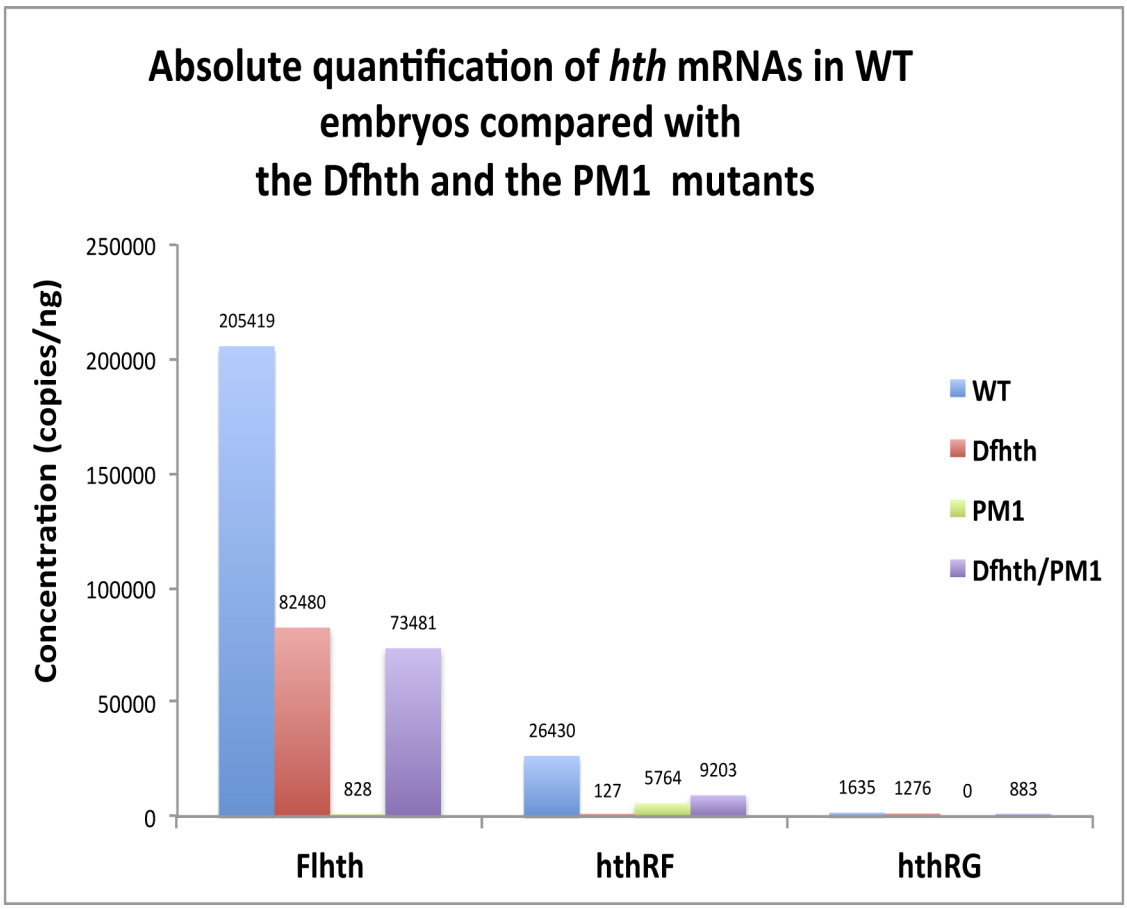


Figure R-13. Molecular characterization of the PM1 mutant. (A) Schematic representation of the P elements 04473 and 06865 (green triangles) used to generate the PM1 deletion in the *hth* genomic region. The arrows indicate the couples of primers used to perform the PCRs shown in B, C and D. **(B)** Amplification of the regions flanking the P elements**.** PCR n.1: amplification using primers of the 04473 and the region outside the deficiency. PCR n.2: no amplification using primers of the 04473 and the region inside the deficiency. PCR n.3: no amplification using primers of the 06865 and the region inside the deficiency. PCR n.4: amplification using primers in the 06865 and outside the deficiency. PCR n.5: positive control of the PCR n.2, performed with the parental fly strain 04473, gives amplification. **(C)** PCRs of the PM1 genomic DNA, compared with the wt. PCR n.6: no amplification of the intron between exons 8 and 9. PCR n.7: amplification using primers of the last intron. PCR n.8: amplification using primers of the last exon. **(D)** RT-PCRs of the PM1 RNA, compared with the wt. PCR n.9: amplification using primers of the last specific exon of the hthRE isoform. PCR n.10: amplification using primers of the last specific exon of the hthRF isoform. PCR n.11: no amplification using primers of the first specific exon of the hthRG isoform. PCR n.12: amplification using primers of the ninth exon and the last one. PCR n. 13: no amplification using primers of the sixth exon and the last one.

R2B Levels of *hth* mRNAs in Dfhth, PM1 and Df/PM1 mutants

Once the PM1 deficiency was molecularly characterized, I decided to check the relative amounts of the different *hth* RNAs in embryos homozygous for the deficiencies as well as in the Dfhth/PM1 heterozygous. To this aim I performed qRT-PCR experiments. The result is summarized in figure R-14.



	WT	Dfhth	PM1	Dfhth/PM1
Flhth	205419	82480	828	73481
hthRF	26430	127	5764	9203
hthRG	1635	1276	0	883

Figure R-14. Levels of *hth* mRNAs in Dfhth, PM1 and Df/PM1 mutants. Dfhth mutants show no hthRF transcript, the levels of the Flhth isoforms are very much reduced and the hthRG shows a mild reduction of 1.2 times. PM1 mutants have very low levels of Flhth isoforms, no hthRG and the hthRF is 5 times less abundant than in wild type. Dfhth/PM1 heterozygous embryos show levels of Flhth similar to those of Dfhth and the levels of hthRF almost double the ones in the PM1 embryos; the amount of hthRG in these embryos is similar to that of the wild type condition. The table below summarizes the values of the histogram.

Dfhth mutants show no hthRF transcript, and the levels of the full-length isoforms are very much reduced. I still observed some Flhth transcripts in these mutants, that I believe correspond to the sum of the maternal RNA (Salvany et al., 2009), truncated Flhth isoforms resulting from the splicing of the last exons (which is not the hthRG) and the remaining hthRG (note that the primers used to amplify the Flhth transcripts also recognize the hthRG, see materials and methods). The hthRG isoform shows a mild reduction of 1.2 times in the mutant background. This result suggests that Dfhth embryos lack a genomic region necessary to transcribe the normal levels of hthRG. As in wild type embryos, I was unable to detect the transcript of the hthRE isoform in any of the mutant combinations.

PM1 mutants show no hthRG, they have very low levels of Flhth isoforms (suggesting that the unexpected RNA found in this mutant is transcribed in little quantities) and the hthRF isoform is 5 times less abundant than in wild type embryos, suggesting that somehow the HD-containing isoforms are required for the normal transcription of this HD-less isoform or that the introns missing in the mutant contain enhancer or regulatory elements.

Dfhth/PM1 heterozygous embryos show levels of Flhth similar to the ones of Dfhth embryos, which should reflect the Flhth that I observe in Dfhth embryos. As expected, hthRF is present in the Dfhth/PM1 embryos, but its levels almost double the ones of the PM1 embryos, indicating that, either the presence of the hthRG in the heterozygous embryos partially rescues the levels of the hthRF transcription, or I have introduced back a genomic region that regulates the levels of hthRF. The amount of hthRG in the Dfhth/PM1 embryos is, as expected, half of that found in the wild type embryos.

R2C Cuticle phenotype of Dfhth, PM1 and Dfhth/PM1 mutants

Hth is necessary for the correct embryo segmentation and, as a Hox cofactor, is required to give identity to the segments along the A/P axis. In order to identify the implication of the distinct Hth isoforms to carry out these functions, I analyzed the cuticle phenotype of the *hth* mutants.

The Dfhth homozygous larvae showed a severe cuticle phenotype (Fig. R-15B). Comparing with the wild type one (Fig. R-15A), indeed, Dfhth larvae present impaired thoracic and abdominal segmentation, and they also show malformations in the head and tail regions.

The PM1 homozygous larvae exhibit less segments than the Dfhth mutants, but better formed. In addition, head and tail regions are better formed than in the Dfhth (Fig. R-15C).

The Dfhth/PM1 larvae have a cuticle phenotype that looks very much like the wild type one. The number of segments is restored and I am only able to detect some malformations in the head (Fig. R-15D). The rescued phenotype of the Dfhth/PM1 embryos suggests that the presence of hthRF and hthRG isoforms is important to have a proper segmentation, even when there are no FlHth isoforms.

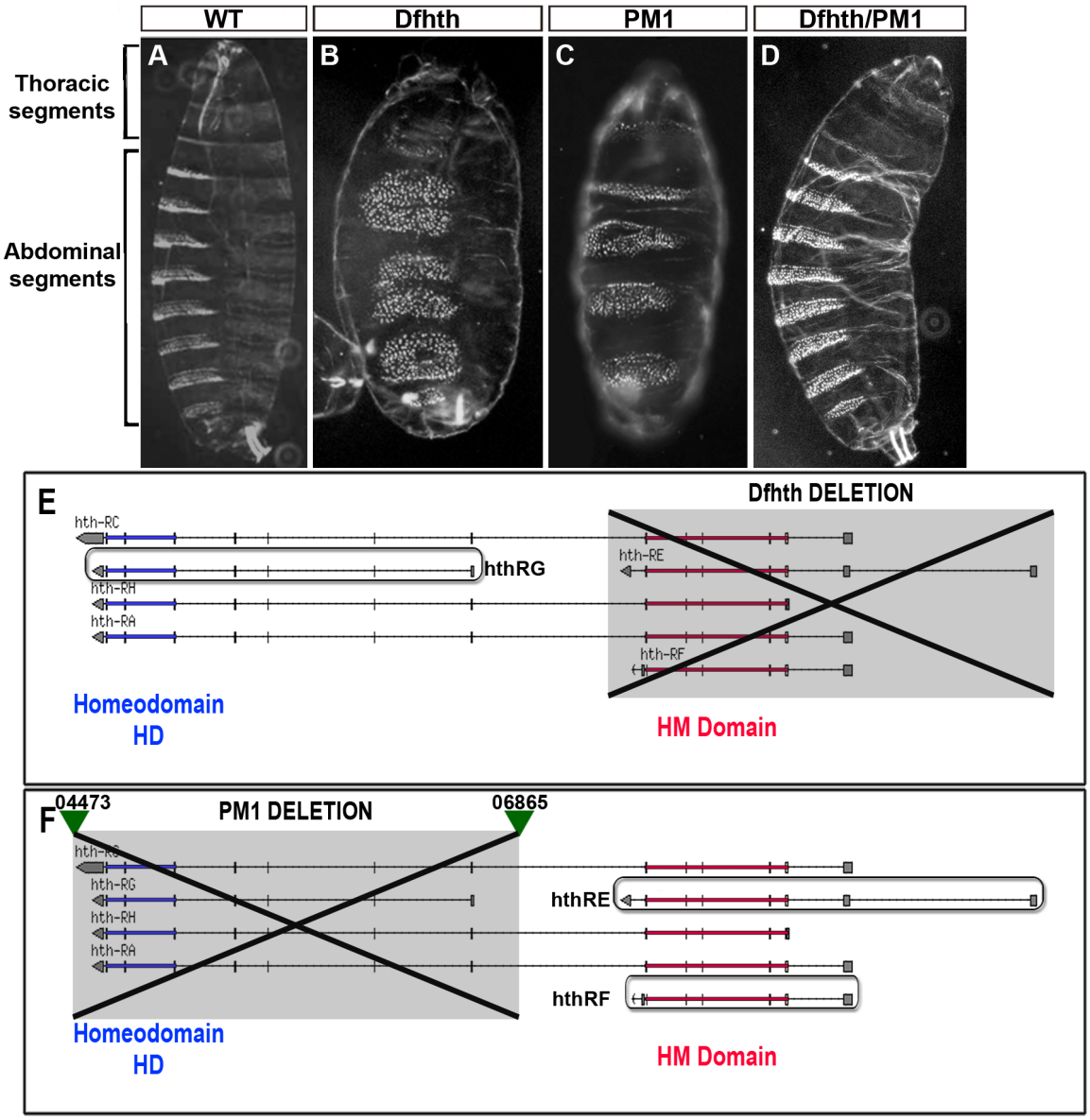


Figure R-15. Cuticle phenotype of Dfhth, PM1 and Dfhth/PM1 mutants and scheme of their deletions. (A) Cuticle phenotype of a wild type larva. **(B)** The cuticle phenotype of a Dfhth larva presents impaired thoracic and abdominal segmentation and malformations in the head and tail regions. **(C)** The cuticle phenotype of a PM1 larva shows few segments and the body extremities are malformed. **(D)** The cuticle phenotype of a Dfhth/PM1 heterozygous larva is similar to the wild type one, as it presents only head malformations. **(E)** The mutants Dfhth lack the 5' genomic region, and in these, only the hthRG isoform can be transcribed. **(F)** The PM1 mutants present a deficiency in the 3' genomic region, caused by the recombination of the P elements 04473 and 06865 (green triangles). In these mutants only the hthRE and hthRF can be transcribed.

R2D Expression of Hth isoforms and Exd in mutant embryos

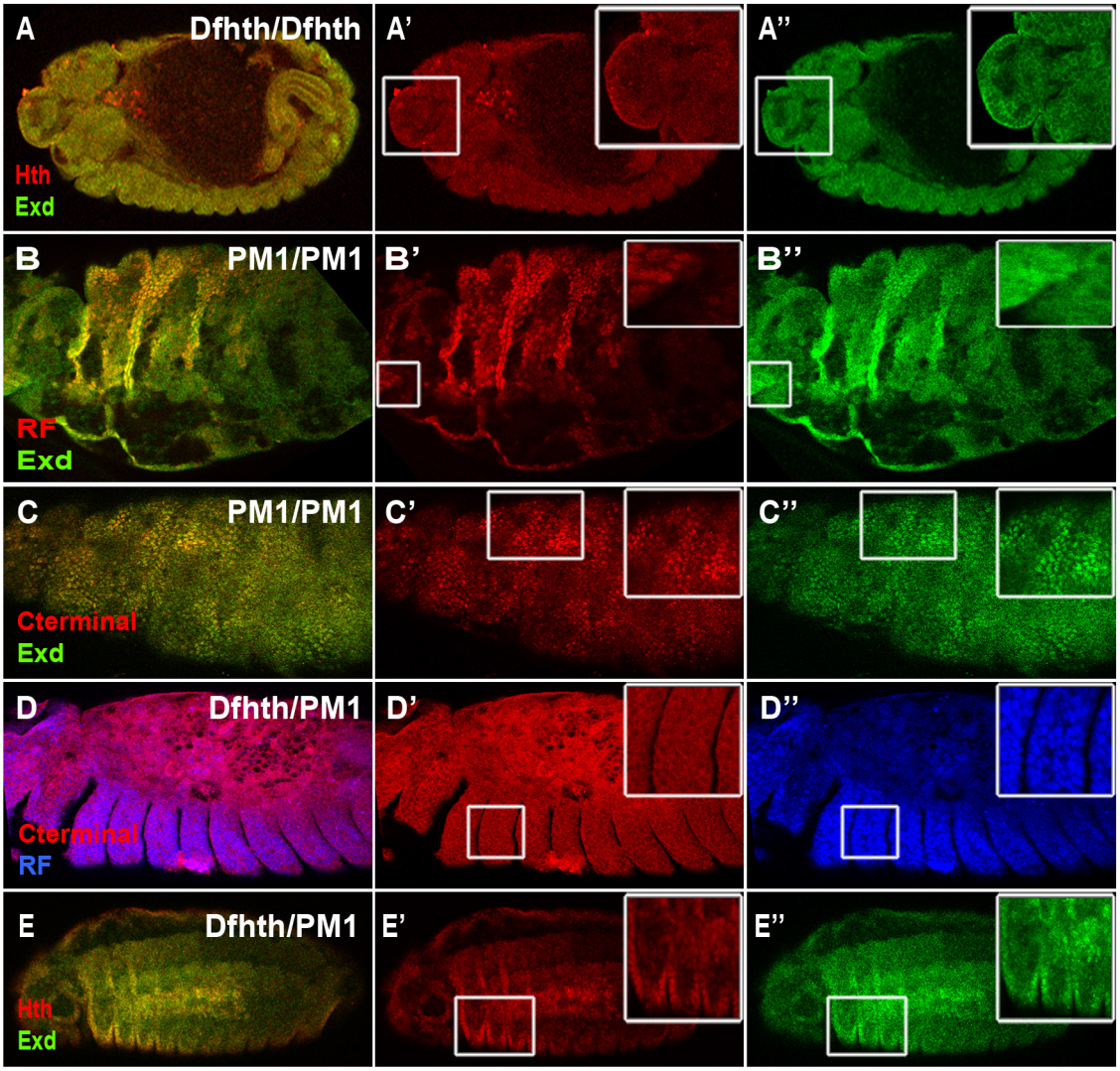
R2Da *In vivo*

In the Dfhth mutants the only isoform present is the HthRG, as the rest of the *locus* is deleted. I can only detect this isoform in the cytoplasm (Fig. R-16A), with the general anti Hth antibody. One possible explanation for this is that HthRG cannot get into the nucleus due to the low levels of its mRNA in these mutants. Another explanation is that HthRG needs the other isoforms for its nuclear translocation. As expected, Exd is cytoplasmic (Fig. R-16A) in these mutants because the HthRG lacks the HM domain, whereby Hth binds Exd and translocates it into the nucleus.

The PM1 mutants show high levels of HthRF expression, especially in the anterior part of the embryo. Exd is mostly cytoplasmic, but it is able to translocate into the nucleus in those cells where the HthRF is nuclear (Fig. R-16B). When I stained these embryos with the specific antibody against the C-terminal part of the protein, I could see a faint cytoplasmic expression, nuclear in some cells. This expression is certainly due to the presence of the short RNA transcribed from the 3' end of the *locus* in these embryos, which then can be translated. The cells that express this aberrant protein in the nucleus also show a nuclear expression of Exd (Fig. R-16C).

The Dfhth/PM1 heterozygous have the three short isoforms and cannot form the FlHth isoforms. When I stained these embryos with the antibody against the C-terminal part of Hth, which corresponds in this case to the HthRG (since in these embryos is the only isoform recognized by this antibody), I could appreciate an expression clearly cytoplasmic, whereas the HthRF is nuclear (Fig. R-16D). In this genetic background Exd is nuclear in those cells where Hth is in the nucleus, but, as in the PM1 mutants, its expression is not normal, as it is weak and vague (Fig. R-16E). In this case I was not able to visualize the aberrant nuclear protein generated in the PM1 mutants. As this is a heterozygous condition, its levels are half of those present in the PM1 embryos, and it could be insufficient to detect the protein with the antibody. Another possibility is that the HthRG could act as a dominant negative, sequestering the aberrant protein in the cytoplasm.

Figure R-16. Expression of the Hth isoforms and Exd in mutant embryos. (A) Localization of Hth and Exd in a Dfhth embryo: the HthRG (red) (the only isoform present in this embryo) and Exd (green) are both cytoplasmic. **(B)** The PM1 mutant embryos show high levels of the HthRF isoform (red) and Exd (green) is nuclear in those cells where the HthRF is nuclear. **(C)** The antibody against the C-terminal part of Hth (red) gives a cytoplasmic expression in the PM1 mutants and is nuclear in few cells, where Exd (green) is also nuclear. **(D)** In Dfhth/PM1 heterozygous embryos the HthRG (red) is cytoplasmic, whereas the HthRF (blue) is nuclear. **(E)** In Dfhth/PM1 heterozygous embryos Exd (green) is nuclear in those cells that show nuclear Hth (red).



R2Db Western blot

I then analyzed which isoforms are present in the mutants by western blot.

Figure R-17A shows the blot hybridized with the general Hth antibody: the band higher than 98 kDa is present in all the mutants. This makes sense as I think that it corresponds to the protein of maternal origin; the bands over the 50 kDa should be absent in all mutants because none of them has the FlHth protein. This result is not clear in the blot. I was also not able to identify the bands corresponding to the HthRE and HthRF isoforms. These two should be absent in the Dfhth embryos but present in wt, PM1 and Df/PM1. The only band that fulfills these criteria is one located below the 64 kDa.

Using the specific antibody against HthRF, I could not verify the presence or absence of this isoform in the mutants (Fig. R-17B). In fact it is not clear if the band located right below the 36 kDa disappears or not in the mutants; the band at 64 kDa is not modified in the mutants and the only band that disappears in PM1 and Dfhth mutants is the one weighting 98 kDa.

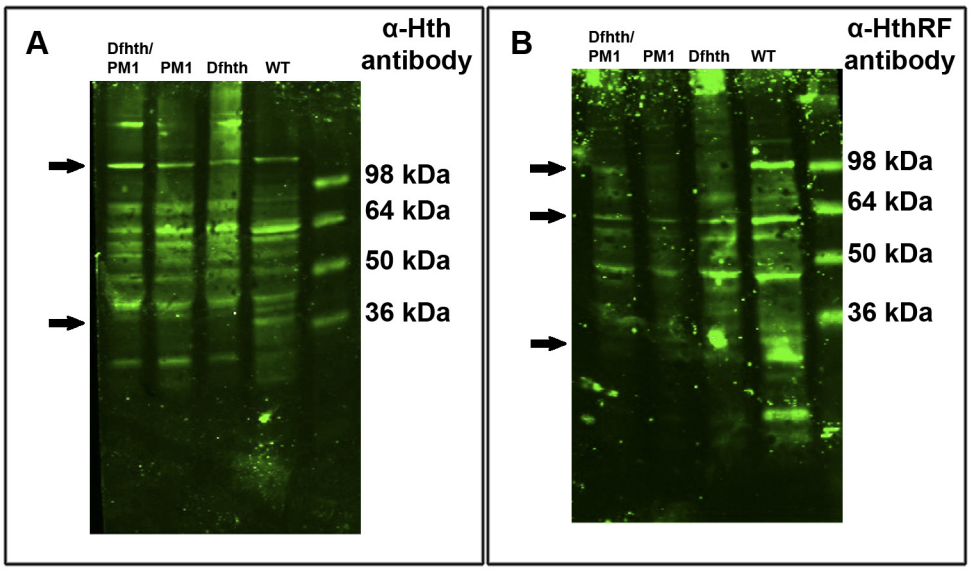


Figure R-17. Hth isoforms present in mutant embryos. (A) The blot revealed with the general antibody against Hth shows a band higher than 98 kDa in all the mutants, which could be the protein of maternal origin. It is not clear if the band over the 50 kDa, corresponding to the FlHth isoforms, disappears in the mutants. The band corresponding to the HthRF, at 36 kDa, should be present in Dfhth/PM1 and PM1 embryos, but it is not clear in the blot. **(B)** Revealing the blot with the specific antibody against the HthRF isoform, it is not clear if the band right below the 36 kDa disappears in the mutants, the band at 64 kDa is not modified and the band at 98 kDa disappears.

R3 Rescue of the Dfhth mutants by the overexpression of Hth isoforms

In order to understand the function of the distinct Hth isoforms, I expressed each of them in a Dfhth mutant background and analyzed the phenotypic outcome. I did these experiments only with this mutant, as it is almost null (only the hthRG can be transcribed), and because, as explained previously, I was not sure about the genomic nature of the deficiency generated in the PM1 mutant.

R3A Rescue of the cuticle phenotype of the Dfhth mutants

As mentioned above, the Dfhth larvae have a severe cuticle phenotype (Fig. R-18B), compared with wild type larvae (Fig. R-18A). I decided to introduce the different Hth isoforms one by one in this mutant background, in order to understand their contribution to embryonic development. I did this experiment by using the nulloGal4 line (Kunwar et al., 2003), which drives the expression of the gene of interest throughout the embryo from early stages of development.

I started off with the overexpression (OE) of the HthRG isoform, as in the Dfhth embryos

it is present at lower levels than in the wild type (Fig. R-14). The OE of the HthRG does not cause any cuticle rescue of the Dfhth larvae (Fig. R-18C), suggesting that the HthRG (even at high levels) is not able to rescue the segmentation phenotype of the Dfhth by itself.

I then introduced the FlHth isoform in the same mutant background and in this case the cuticle phenotype of the larvae was almost totally rescued (Fig. R-18D). There were still some defects, mostly at the anterior and posterior of the larva.

Expressing the short HthRE isoform in the Dfhth embryos, I obtained a partial rescue of their cuticle phenotype, as the number of the segments gets normal, but their morphology is still aberrant. The head is not rescued (Fig. R-18E).

Finally, I introduced the short HthRF isoform back in the Dfhth embryos. In this experiment I achieved a good rescue, as the number and the morphology of the body segments were normal (Fig. R-18F). The first abdominal segment was not completely rescued, suggesting that the normal function of the Ubx protein was not totally reestablished. I observed also some defects in the thoracic segments, in the head and in the posterior part of the larva.

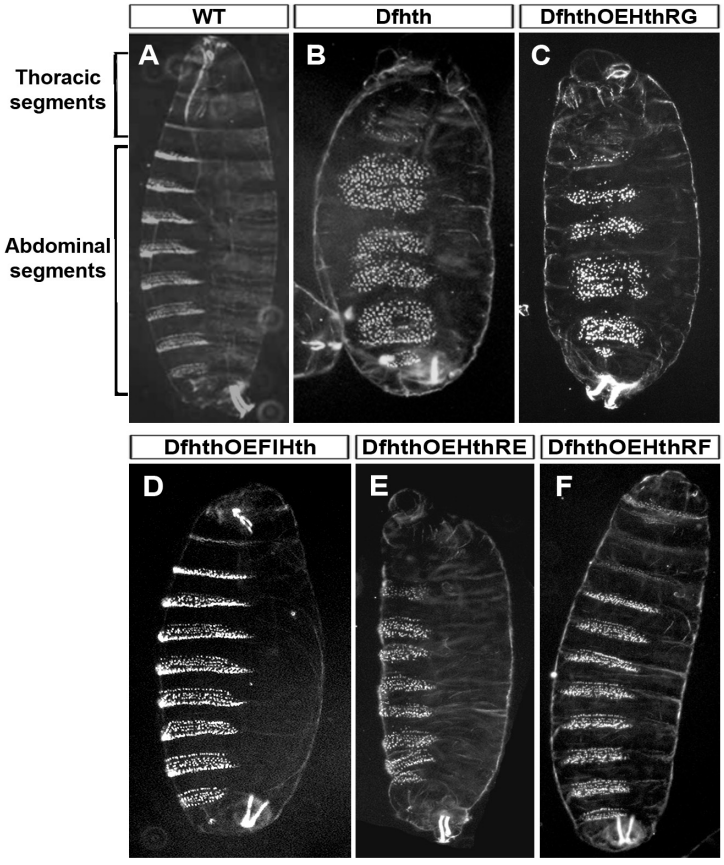


Figure R-18. Cuticle phenotype of a Dfhth larva and degree of rescue of the distinct isoforms in this mutant background. (A) Cuticle phenotype of a wild type larva. **(B)** The abnormal cuticle of the Dfhth mutant, showing severe defects in body segmentation, and in the head and tail regions. **(C)** The overexpression of the HthRG does not lead to any rescue of the Dfhth mutant. **(D)** The introduction of the FlHth isoform results in an almost complete rescue of the phenotype. **(E)** The expression of the HthRE causes a rescue of the number of segments, but not of their morphology. **(F)** The introduction of the HthRF leads to a good rescue, in the number and in the morphology of the segments.

R3B Expression of Hth and Exd in the rescued embryos

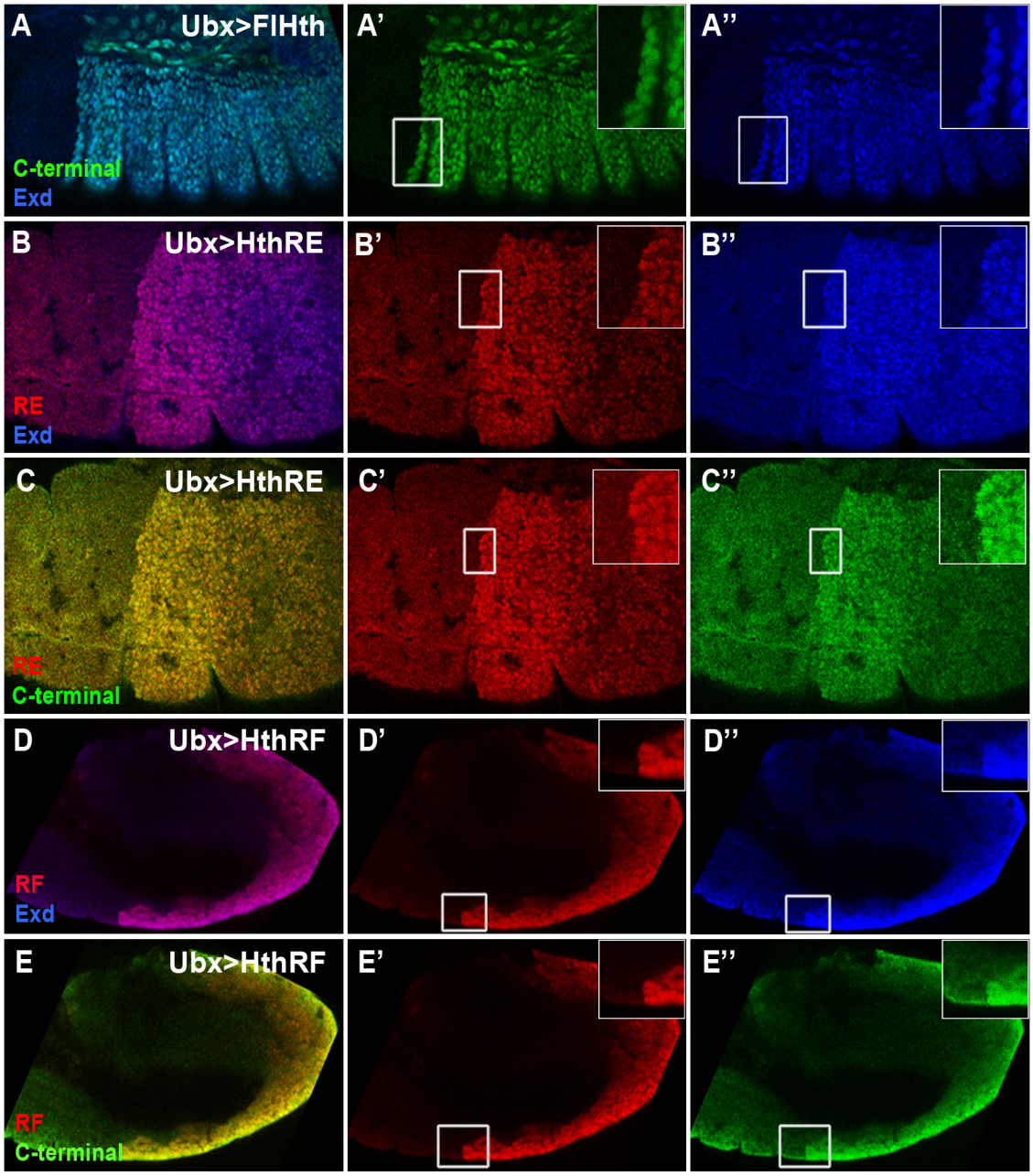
I then checked the distribution of the different Hth isoforms and of Exd in the OE experiments. For this experiment I used the UbxGal4 line (Herranz and Morata, 2001; de Navas et al., 2006) in order to compare the OE domain of the embryo with the region where there is no OE.

When I expressed the FlHth isoform in the Ubx domain of the Dfhth embryos, the isoform introduced was nuclear and Exd was able to translocate into the nucleus (Fig. R-19A). I could not analyze the distribution of the HthRG isoform, as the antibody against the C-terminal part of Hth recognizes both the FlHth (which is the one that I was expressing) and the HthRG.

The expression of the HthRE isoform in the Ubx domain of the Dfhth mutants leads to the nuclear translocation of Exd, probably due to the presence of an Hth isoform containing the HM domain (Fig. R-19B). In fact the HthRE is nuclear and I also detect nuclear HthRG, recognized by the antibody against the C-terminal part of Hth (Fig. R-19C).

The expression of the HthRF isoform in the Dfhth embryos results in its nuclear distribution (Fig. R-19D) and, as observed for the HthRE isoform (Fig. R-19B and R-19C), this leads to the nuclear translocation of Exd and the HthRG isoform (Fig. R-19D and R-19E).

Figure R-19. Expression of the distinct Hth isoforms in the Ultrabithorax (Ubx) domain of the Dfhth mutants. (A) The expression of the FlHth isoform (green) in the Ubx domain induces the nuclear translocation of Exd (blue). **(B)** The introduction of the HthRE causes the nuclear translocation of this isoform (red) and Exd (blue). **(C)** The expression of the HthRE (red) causes the nuclear translocation of the HthRG (green). **(D)** The OE of the HthRF (red) leads to the nuclear translocation of Exd (blue). **(E)** The OE of the HthRF (red) also leads to the nuclear translocation of HthRG (green).



R4 Cross-regulatory interactions of the different Hth isoforms

The results obtained during my work led me to consider *hth* as a complex *locus*, which does not give rise to a unique protein, but rather to different proteins that presumably play distinct roles. Given this complexity, I wondered if the different Hth isoforms could regulate each other. To test this hypothesis I overexpressed one isoform in a wild type embryo and checked the expression of the other ones. For this experiment I used again the UbxGal4 line, since it allows the comparison between the wild type region of the embryo and the overexpression domain. The Ubx expression domain is visible in figure R-20A'''.

As shown in figure R-20B, embryos with the genotype UbxGal4/UASFlHth display lower

levels of the HthRF isoform in the Ubx domain. This observation indicates that, when overexpressed in the embryo, FlHth is able to repress or destabilize the HthRF isoform (compare Fig. R-20B'' with R-20A'').

The overexpression of the HthRE with the same driver also leads to a downregulation of the HthRF (Fig. R-20C), but the levels of the HD-containing isoforms do not change significantly (Fig. R-20D).

The overexpression of the HthRF does not cause any significant effect on the levels of the other isoforms, but I should note that in this case I was not able to detect clearly the higher accumulation of this isoform in the Ubx domain, suggesting that probably its ectopic expression is not stable enough to regulate the other isoforms (Fig. R-20E).

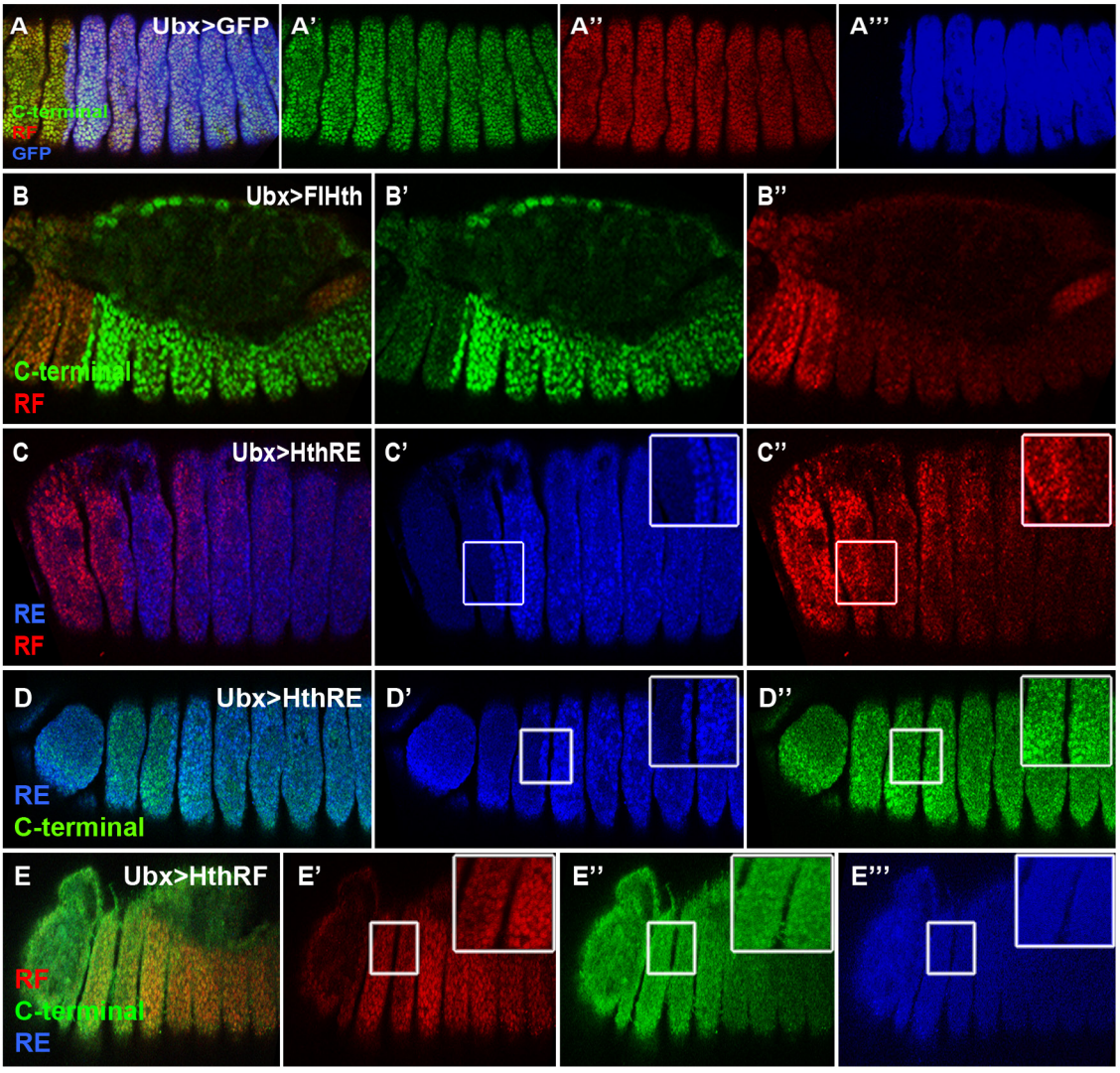


Figure R-20. Cross-regulation of the different Hth isoforms in wild type embryos. (A) Normal distribution of FlHth (green) and HthRF (red) when overexpressing GFP in the Ubx domain (GFP in blue). **(B)** The OE of the FlHth isoforms in the Ubx domain (green) causes the reduction of the HthRF isoform levels (red). **(C)** The OE of the HthRE (blue) isoform also leads to the diminishment of the HthRF isoform levels (red). **(D)** The OE of the HthRE isoform (blue) does not cause any significant change of the levels of the HD-containing Hth isoforms (green). **(E)** The OE of the HthRF isoform (red) does not lead to any change of the levels of the HD-containing Hth isoforms (green) and of the HthRE isoform (blue).

R5 The full-length Hth isoform activates *exd* transcription

As mentioned in the introduction, Hth acts as a cofactor of the Hox proteins together with the homeodomain-containing protein Exd. In the past, several groups stated that the nuclear translocation of Exd is due to Hth. Indeed, Hth has a conserved domain called HM, located in the N-terminal part of the protein, through which it binds Exd. All the Hth isoforms, except the HthRG, have the HM domain.

The observation that in the early blastodermic embryos the HthRF is nuclear, whereas Exd stays in the cytoplasm (Aspland and White, 1997) led me to guess that probably the common assumption Hth+Exd translocating together to the nucleus is not correct.

Besides, I observed that Exd appears in the nuclei shortly after the HD-containing isoforms start to be nuclear. For this reason I reasoned that perhaps Hth could be able to transcriptionally activate its partner Exd.

To verify my hypothesis, I ectopically expressed the FlHth isoform in the wing pouch, where *exd* mRNA have been reported to be homogeneous and cytoplasmic (Rauskolb et al., 1995). To perform this experiment I used the salGal4 line (Cruz et al., 2009), which drives the ectopic expression of the gene of interest in the wing pouch.

When I ectopically expressed the FlHth isoform in the wing pouch, the levels of *exd* mRNA are drastically increased (compare Fig. R-21A with R-21B) and where the FlHth isoform is ectopically expressed, the Exd protein becomes nuclear (Fig. R-21C). The nuclear Exd in the wing pouch is not the result of a simply translocation of its wild type levels in this domain, as the levels of the protein are much higher than the ones normally present in the cytoplasm of these cells. This result led me to conclude that Hth, probably in addition to translocate Exd into the nucleus, is able to activate its transcription.

I performed a similar experiment in the leg imaginal disc. Forcing expression of FlHth in the *bric a brac* (*bab*) domain induces activation of *exd* mRNA (compare Fig. R-21D with R-21E) and high levels of nuclear Exd protein.

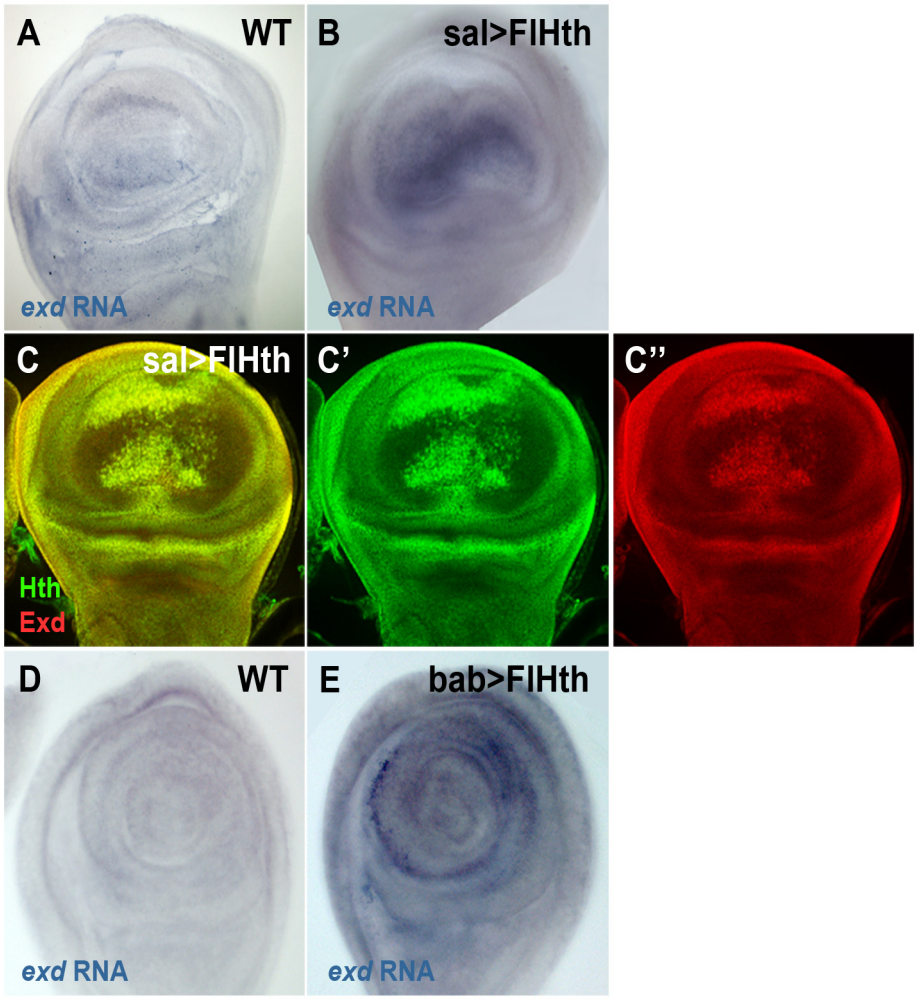


Figure R-21. FlHth isoform activates *exd* transcription. (A) In the wing pouch, the levels of *exd* mRNA are homogeneous in wild type conditions. **(B)** When the FlHth is ectopically expressed in the *spalt* domain, *exd* transcription is activated, and the levels of *exd* mRNA are increased. **(C)** As a consequence of this activation, the levels of Exd protein (red) are higher and its expression is nuclear. **(D)** Wild type expression of *exd* mRNA in the leg disc. **(E)** Expression of FlHth in the *bric a brac* (*bab*) domain of the leg disc activates *exd* transcription.

V. DISCUSSION

DISCUSSION

D1 The distribution of Hth isoforms in embryos and cell lines suggests that they have distinct functions

The quantification of the *hth* mRNAs was previously done by Glazov et al. in 2005, but they didn't take into account all the transcripts, as the hthRF and the hthRG were still unknown. However, in contrast to my results, they were able to detect very low levels of the hthRE that I was not able to see. Although the primers used for the quantification experiments annealed in the same final exon in both experiments, they were not exactly the same. This could be the reason for the discrepancy between the results.

The fluorescent *in situ* hybridization experiments demonstrate that this technique is not appropriate to study the distribution of the *hth* isoforms. Noro et al. used this method to visualize the *hth* RNAs in 2006. They performed a visible *in situ* hybridization that does not distinguish between the nuclear dots and the cytoplasmic distribution. Indeed, they improperly inferred that the distinct *hth* RNAs colocalize in the embryo.

In any case, we can conclude that the short hthRE isoform is present in very low amounts in the embryo. Despite its very low levels, this mRNA is translated into protein, as I am able to visualize it with the specific antibody generated against this isoform. The hthRE could have a "homeostatic function" in the embryo and it could be transcribed at higher levels in the larval or adult stage. The HthRE is clearly detectable in both cell lines and it seems to be secreted, as it localizes in the Golgi and sometimes it is detectable on the cell membranes or right outside the cells. It can result surprising that a transcription factor could be secreted, but recently more HD proteins have been reported to diffuse out of the cell (Prochiantz and Joliot, 2003; Layalle et al., 2011), suggesting a novel common function of these transcription factors, that in the case of *hth*, it has been adapted to one HD-less isoform. These observations support the hypothesis of the homeostatic function, as the embryo cells could fulfill this role by secreting the HthRE. As it is not possible to visualize a such detailed expression of this protein in the embryo, due to the size of its cells, I am only able to see a general and low expression of the HthRE throughout the embryo development with the specific antibody.

The observation that the Hth isoforms are not always expressed in the same cells throughout embryo development, suggests that they may not have the same function. The downregulation of the HthRF isoform by stage 9 in the abdominal segments could be dependent on Hox proteins. Actually it is known that general Hth expression in the abdominal segments starts to decline by stage 11 and that this repression is exerted by the abdominal Hox proteins (Rauskolb et al., 1993; Azpiazu and Morata, 1998; Kurant et al., 1998). The high expression of Hth seen in the thoracic segments at these stages is actually the sum of the HthRF and the HD-containing isoforms. Because HthRF is repressed in the abdominal segments and the

others are expressed at uniform levels all over the embryos, the result is the decline toward the posterior segments observed with the general antibody.

The HthRF colocalizes in many cells with the HD-containing isoforms, suggesting that they could form a heterodimer and the HthRF could stabilize the complex of transcription factors bound to the DNA.

It is worth noticing that the HthRE and the HthRF differ only in the last 24 aminoacids, but this seems to be essential for their different functions. Both proteins have the same number of aminoacids (266), and in positions 256-259 (in the divergent part) they have the same aminoacids (ASY). The biological significance of this observation is unknown. The specific exons of the HthRE and the HthRF are highly conserved in all dipterans, suggesting that HD-less Hth isoforms may exert conserved functions in this order of insects (Noro et al., 2006). In vertebrates the transcription of HD-less variants also occurs in the *meis* gene, underscoring the functional significance of these isoforms in animal development (Hyman-Walsh et al., 2010; Crist et al., 2011).

It is not surprising that the Flhth mRNAs are the most abundant ones, as the levels obtained in the qPCR experiments are the sum of four distinct mRNAs (three full-length and the hthRG) that I could not distinguish, as well as the RNA of maternal origin (Salvany et al., 2009).

The HD-containing isoforms are mostly cytoplasmic in the S2 cells with an additional dotted expression in the nuclei. However, this expression is different in the DmD-8 cells. In there, they exhibit a ubiquitous distribution in the nucleus, pointing to a different role of these proteins depending on the tissue where they are expressed. The S2 cells have an embryonic origin, whereas the DmD-8 cells are derived from wing discs.

The analysis of the expression of the Hth isoforms in the embryos by western blot reveals proteins with different weight than the predicted ones, indicating that probably these isoforms go through several post-translational modifications that could contribute to the functional diversity of these (Stamm et al., 2005). Indeed, many putative myristoylations, phosphorylations and amidations sites have been predicted for the isoforms (Fig. R-4).

In my western blot analysis I distinguish a different pattern of bands in S2 cells and embryos when hybridizing with the specific antibodies against HthRF and HthRE. This result suggests that these isoforms are differentially modified depending on the embryonic stage. The S2 cell line is derived from a primary culture of late stage (20-24 hours old) embryos (Schneider, 1972), whereas the embryos used to do the western blot were collected from all the stages. The Hth isoforms could be modified to perform specific functions in the late embryonic development, whereas they may not be subject to modifications to fulfill more general roles in the early development, like the assembly of heterochromatin structures.

D2 The different cuticular phenotype of *hth* mutants points to distinct roles of the Hth isoforms in segmentation

In Dfhth mutant embryos the only Hth protein detected is cytoplasmic and the cuticle phenotype of these larvae is very strong. With the qPCR experiments I could verify that in these mutants the only normal mRNA transcribed is the hthRG. In the Dfhth mutants, indeed, the levels of the Flhth transcripts correspond to the hthRG isoform, to the maternal RNA and probably to truncated RNAs resulting from the splicing of the last exons. The reduced levels of hthRG suggest that the absence of the other short isoforms or the Flhth transcripts could affect the transcription of the hthRG or that regulatory elements have been deleted in this mutant.

These results also indicate that the cytoplasmic HthRG, by itself, cannot assure the correct embryo segmentation in the absence of the other isoforms. This phenotype is not due to the reduced levels of HthRG, because the increase of these levels does not rescue the larval phenotype. The HthRG could have a cytoplasmic function in wild type embryos or it could need the presence of the other isoforms to enter the nucleus. The fact that the HthRG is cytoplasmic also in the Dfhth/PM1 embryos, that have HthRF and HthRE, leads me to guess that this isoform cannot enter the nucleus and that it could have specific functions in the cytoplasm, possibly independent of the homeotic proteins. This could give an explanation for the severe cuticle phenotype of the Dfhth mutants, as the HthRG does not act as a Hox cofactor, and so it is unable to ensure a proper segment identity. The aberrant segmentation of these mutants could also be due to the absence of interaction between the segment polarity protein Engrailed and its cofactor Hth (previously reported by Kobayashi et al., 2003).

The cuticle phenotype of the PM1 mutants indicates that the short isoforms HthRE and HthRF contribute to the correct morphogenesis of the body segments, but they are not sufficient to ensure a proper body segmentation.

The levels of mRNA that I detect in the PM1, when using specific primers for the last exons, should correspond to the short aberrant RNA resulted from the improper recombination. It is worth noticing that in these mutants, the levels of maternal RNA are very low, suggesting that the deleted genomic region contained a regulatory sequence responsible for the expression of this RNA. The PM1 mutants also display less hthRF than their wild type siblings, again suggesting that there were regulatory elements in the missing introns. Indeed, the introns deleted in the PM1 mutant are the most conserved ones in the *hth*/*meis* evolution throughout species (Irimia et al., 2011), and usually a high conservation of the sequences reflects a function of these. Another explanation of this result could be that the Flhth mRNAs or proteins are necessary for the normal transcription of hthRF. I do not favor this hypothesis, because I have demonstrated that in wild type conditions overexpression of FlHth represses HthRF, suggesting that Flhth has a repressive role over hthRF. In addition, I was also able to show that in the heterozygous mutants Dfhth/PM1 the levels of hthRF are double as much

as in PM1. These heterozygous conditions do not form FlHth proteins, so the presence of the hthRG and/or regulatory sequences restores partially the normal levels of the hthRF transcript.

The cuticle phenotype of the Dfhth/PM1 heterozygous larvae points to a requirement of the short isoforms for the proper segmentation (as observed in the PM1 mutant) and to a need of the FlHth isoforms for the correct formation of the head. It is surprising that the Dfhth/PM1 embryos have an almost completely rescued cuticular phenotype, specially compared to the PM1 mutants. The two mutant conditions differ in the presence of the HthRG, which does not seem to have a role in the embryonic segmentation, and in the intronic regions that are missing in the PM1. It may be possible that the combination of the three short isoforms could reestablish the proper segmentation, or that in the Dfhth/PM1 embryos the intronic regulatory sequences do so.

The identification of the Hth isoforms in the mutants by western blot analysis is not concluding, as only the presence of the maternal protein is identifiable in all the mutants. I think that the results obtained in the blots relative to the mutants are so confusing due to the cross-regulatory interactions between the isoforms. Indeed, the absence of one or more isoforms leads to changes in the levels of the remaining isoforms. Furthermore, as all the isoforms present several predicted post-translational modifications sites, they could be differently modified in the distinct mutants, and this makes the isoforms arduously identifiable in the blots.

D3 The presence of different combinations of Hth isoforms results in distinct phenotypic outcomes of the Dfhth mutants

The increase of the levels of the HthRG in the Dfhth mutants does not cause any rescue to the larvae, indicating that the HthRG alone, even at high levels, is not able to ensure a proper segmentation.

The introduction of the FlHth isoforms back to the same mutant background leads to a good rescue of the cuticle phenotype as the larvae show only defects in the head and tail regions. These embryos present nuclear FlHth and nuclear Exd indicating that the FlHth isoforms, translocating Exd into the nucleus, cause the reestablishment of the correct segmentation of the embryo and that the HD-less isoforms are required for the proper formation of the extremities, or that the Gal4 line utilized is not sufficiently expressed there.

The introduction of the HthRE back to the Dfhth mutants leads to the formation of the correct number of segments with abnormal morphology, and does not cause the rescue of the head and tail formation. In these embryos the HthRE, the HthRG and Exd are nuclear, indicating that somehow high levels of HthRE lead to the nuclear translocation of the HthRG

and Exd and this causes a partial rescue of the embryo.

When I introduce HthRF to the Dfhth mutants the rescue of the cuticle phenotype is very good, as the only defects observed lie in the head and tail regions. The HthRF, the HthRG and Exd are nuclear in these embryos, indicating that the presence of these proteins in the nucleus leads to the phenotypic rescue of the embryos.

It is surprising that in the Dfhth/PM1 embryos, where the HD-less isoforms are present, the HthRG stays in the cytoplasm, whereas it becomes nuclear with the expression of the same isoforms in the Dfhth mutants. The HthRG could be cytoplasmic in wild type conditions and the high levels of the HD-less isoforms expressed in the Dfhth could translocate the HthRG into the nucleus. Alternatively, the HthRG could be nuclear in wild type conditions and it may need the Flhth isoforms to get there; for this reason the Dfhth/PM1 embryos present cytoplasmic HthRG. It will be interesting in the future to find out a way to discriminate between the expressions of the two isoforms (HthRG and FlHth) to be able to distinguish between these two possibilities.

D4 The cross-regulatory interactions between the Hth isoforms assure the proper levels of Hth during embryonic development

The existing cross-regulatory interactions between the Hth isoforms is not surprising, as a gene with so many pivotal roles during development must be strictly regulated. This is one of the ways by which the cell ensures the correct outcome of the alternative splicing, assuring the right relative quantities of each isoform. This cross-regulation between isoforms has already been found in mammals for the human prolactin receptor (Tan and Walker, 2010), but this is not the only way by which the alternative splicing is regulated. Actually, it has been found that the levels of the different isoforms of the same given protein are conserved between different individuals, but it is strictly tissue-specific and age-dependent (Chisa and Burke, 2007). These data suggest that the selection of the mRNA splice-isoform is subject to a genetic and an epigenetic control.

The *hth locus* could be subject to several levels of regulation. The hthRE isoform presents a long 5' UTR (Fig. I-10B) and a noncoding antisense RNA overlaps with this sequence (Flybase). Moreover, the *hth* mRNA is the predicted target of 23 microRNAs (reported in the microRNA database), and a microRNA is transcribed from the last intron of *hth*, whose target is unknown. Furthermore, the respective specific exons of the HthRE and the HthRF have a long untranslated sequence, which could allow further ways of regulation. Actually, the untranslated sequence of the hthRF is highly conserved among diptera and it has been shown to play a central role in the regulation of *hth* pre-mRNA splicing (Glazov et al., 2006). Finally, *hth* presents very long introns and four of them are highly conserved among all the species (Irimia et al., 2011), and it has been found that in humans, reduced expression of the *meis1*

gene, through intronic cis-regulatory elements, predisposes to RLS (Xiong et al., 2008). All these data point to important regulatory functions of the intronic regions of the *hth*/*meis* gene.

Therefore, the alternative splicing is a highly regulated mechanism, that could depend on the post-translational modifications of the different isoforms in a given tissue, on the presence of intronic regulatory sequences, on the microRNA-dependent regulation, and on the presence of untranslated non coding RNAs overlapping with the coding sequences of one or more isoforms.

It is important to take into account the cross-regulation between the Hth isoforms to avoid improper deductions from overexpressing experiments.

D5 A careful observation of the effects of Hth on the subcellular localization of Exd, reveals a novel aspect of theis relationship

The ectopic expression of the FlHth isoform in domains of the wing and the leg discs leads to the transcriptional activation of *exd*. The increase of the *exd* mRNA levels leads to an increment of the Exd protein and to its nuclear translocation. Therefore FlHth, probably in addition to facilitate the nuclear translocation of Exd, is able to activate its transcription. These data explain why Exd starts to be nuclear when the HD-containing isoforms appear in the nucleus, actually short before the germ-band extension (Aspland and White, 1997). When the HD-less HthRF isoform starts to be nuclear, in the early blastodermic embryo, Exd stays in the cytoplasm. This observation indicates that, although this HM-containing isoform is expressed in the nucleus, it does not translocate Exd there, and also shows that, even having the HM domain, HthRF does not translocate Exd to the nucleus. The critical aspect for the nuclear translocation of Exd seems to be the high levels of Exd. Rivas et al. observed in 2013 that in some cells of the abdominal segments 8 and 9 Exd stays in the cytoplasm despite the nuclear localization of Hth. The Hth expression that they observed could be due to the HthRF, unable to activate the *exd* transcription. In the Dfhth embryos Exd is cytoplasmic, as the HthRG does not get the nucleus and therefore it cannot activate its transcription. In the PM1 mutants Exd is nuclear in the cells where the aberrant protein recognized by the anti C-terminal antibody is nuclear. This observation suggests that this abnormal Hth protein, that has an HD, is able to activate *exd* transcription, but in a less efficient manner, as the Exd expression in these cells is always lower than in the wild type. Actually Hth could act, through the full-length isoforms, only as a transcriptional activator and possibly it is not responsible of the nuclear translocation of Exd. Indeed, Abu-Shaar et al. observed in 1999 that, even in the absence of Hth, Exd gets into the nucleus if expressed at high levels. This observation suggests that the critical point for the nuclear translocation of Exd is its protein levels. Hth could be therefore responsible for activating *exd* to allow these high protein levels. Inbal et

al. in 2001 discovered that Hth acts as a transcriptional activator in many developmental processes, through its HD, independently of the nuclear localization of Exd. Hyman-Walsh et al. shows in 2010 that a Meis isoform presents a domain of transcriptional activity right after the HD, whose biological function is still unknown.

My work points to a different expression and function of the Hth isoforms which is also being supported by results obtained in other species, like the discovery that in humans the HD-less isoform of Meis1 is downregulated in colorectal cancer (Crist et al., 2011) or the founding that also in chicken the *meis* genes give rise to different isoforms which have a distinct pattern of expression during embryogenesis (Sanchez-Guardado et al., 2011).

Further researches need to be done to investigate the specific functions of all the *meis*/*hth* isoforms, to elucidate their respective implications in normal development and hematopoiesis, and therefore how their abnormal expression give rise to acute diseases as leukemias and tumors.

VI. CONCLUSIONS

CONCLUSIONS

1. The *hth* mRNAs are transcribed at distinct levels in *Drosophila* wild type embryos. The more represented mRNAs are the three full-length ones, followed by the hthRF and the hthRG. The levels of the hthRE are not detectable with our techniques.

2. The Hth protein isoforms are differentially distributed during *Drosophila* embryonic development. The first isoform that translocate into the nucleus is the HthRF in the early blastodermic embryo, at the time Exd is still cytoplasmic. The HD-containing isoforms get into the nucleus later, short before germ-band extension. By stage 9 the HthRF isoform starts to be downregulated in the abdominal segments of the embryo.

3. The Hth isoforms are differentially distributed in S2 and DmD8 *Drosophila* cell lines, pointing to a different role of these proteins depending on the tissue where they are expressed. The HthRE isoform is localized in the Golgi apparatus of the S2 and DmD8 cell lines and seems to be secreted.

4. All the Hth isoforms seem to be post-translationally modified in embryos and cell lines, although not all the isoforms show the same modifications in both systems, suggesting that the post-translational modifications play important roles in development.

5. The Dfhth and PM1 mutants show a severe cuticle phenotype and the heterozygous Dfhth/PM1 present a phenotype more similar to the wild type one, suggesting that the combination of the three short isoforms could reestablish the proper segmentation.

6. The overexpression of the HthRG isoform in the Dfhth mutants does not lead to any rescue of the cuticle phenotype. The introduction of the HthRE causes a slight rescue of the Dfhth phenotype. The FlHth and the HthRF isoforms lead to a good rescue of the Dfhth phenotype.

7. In the Dfhth mutants the HthRG, even when overexpressed, is not able to translocate into the nucleus. The FlHth, HthRE and HthRF isoforms expressed in the Dfhth mutants lead to nuclear localization of Exd and HthRG.

8. The ectopic expression of the FlHth and the HthRE isoforms in wild type embryos causes a downregulation of the HthRF isoform. The ectopic expression of the HthRE isoform in wild type embryos does not have any significant effect on the HD-containing Hth isoforms. The ectopic expression of the HthRF isoform in wild type embryos does not have any significant effect on the other Hth isoforms.

9. The ectopic expression of the FlHth isoform in the *spalt* domain of the wing imaginal disc and in the *bric a brac* domain of the leg imaginal disc activates *exd* transcription, leading to an increment of the Exd protein. The high levels of Exd result in its nuclear translocation.

VII. REFERENCES

REFERENCES

Abu-Shaar, M. and Mann, R. S. (1998) 'Generation of multiple antagonistic domains along the proximodistal axis during *Drosophila* leg development', *Development* 125(19): 3821-30.

Abu-Shaar, M., Ryoo, H.-D. and Mann, R. S. (1999) 'Control of the nuclear localization of Extradenticle by competing nuclear import and export signals.', *Genes and Development* 13: 935-945.

Adams, M. D., Celniker, S.E. y cols (2000) 'The genome sequence of *Drosophila melanogaster*', *Science* 287(5461): 10.

Aldaz, S., Morata, G. and Azpiazu, N. (2005) 'Patterning function of homothorax/extradenticle in the thorax of Drosophila', *Development* 132(3): 439-46.

Aspland, S. E. and White, R. A. H. (1997) 'Nucleocytoplsmic localisation of extradenticle protein is spatially regulated throughout development in *Drosophila*.', *Development* 124(3): 741-747.

Azpiazu, N. and Morata, G. (1998) 'Functional and regulatory interactions between Hox and extradenticle genes', *Genes & development* 12(2): 261-73.

Azpiazu, N. and Morata, G. (2000) 'Function and regulation of *homothorax* in the wing imaginal disc of *Drosophila*.', *Development* 127: 2685-2693.

Azpiazu, N. and Morata, G. (2002) 'Distinct functions of homothorax in leg development in Drosophila', Mechanisms of development 119(1): 55-67.

Berthelsen, J., Zappavigna, V., Ferretti, E., Mavilio, F. and Blasi, F. (1998a) 'The novel homeoprotein Prep1 modulates Pbx-Hox protein cooperativity.', *The EMBO journal* 117(5): 1434-1445.

Berthelsen, J., Zappavigna, V., Mavilio, F. and Blasi, F. (1998b) 'Prep1, a novel functional partner of Pbx proteins.', *The EMBO journal*: 1423-1433.

Bessa, J., Gebelein, B., Pichaud, F., Casares, F. and Mann, R. S. (2002) 'Combinatorial control of Drosophila eye development by eyeless, homothorax, and teashirt', *Genes & development* 16(18): 2415-27.

Brand, A. H. and Perrimon, N. (1993) 'Targeted gene expression as a means of altering cell fates and generating dominant phenotypes.', *Development* 118: 401-415.

Bürglin, T. R. and Ruvkun, G. (1992) 'New motif in PBX genes', *Nature genetics* 1(5): 319-20.

Bürglin, T. R. (1994) 'A Caenorhabditis elegans prospero homologue defines a novel domain', *Trends in biochemical sciences* 19(2): 70-1.

Bürglin, T. C. (1997) 'Analysis of TALE superclass homeobox genes (MEIS, PBX, KNOX, Iroquois, TGIF) reveals a novel domain conserved between plants and animals.', *Nucleic acids research* 25: 4173-4180.

Bürglin, T. R. (1998) 'The PBC domain contains a MEINOX domain: coevolution of Hox and TALE homeobox genes?', *Development genes and evolution* 208(2): 113-6.

Calvo, K. R., Knoepfler, P., McGrath, S. and Kamps, M. P. (1999) 'An inhibitory switch derepressed by pbx, hox, and Meis/Prep1 partners regulates DNA-binding by pbx1 and E2a-pbx1 and is dispensable for myeloid immortalization by E2a-pbx1', *Oncogene* 18(56): 8033-43.

Carroll, S. B., DiNardo, S., O'Farrell, P. H., White, R. A. and Scott, M. P. (1988) 'Temporal and spatial relationships between segmentation and homeotic gene expression in Drosophila embryos: distributions of the fushi tarazu, engrailed, Sex combs reduced, Antennapedia, and Ultrabithorax proteins', *Genes & development* 2(3): 350-60.

Casares, F., Mann, Richard S. (1998) 'Control of antennal versus leg development in *Drosophila*', *Nature* 392: 4.

Casares, F. and Mann, R. (2000) 'A dual role for *homothorax* in inhibiting wing blade development and specifying proximal wing identities in *Drosophila*.', *Development* 127: 1489-1498.

Caslini, C., Yang, Z., El-Osta, M., Milne, T. A., Slany, R. K. and Hess, J. L. (2007) 'Interaction of MLL amino terminal sequences with menin is required for transformation', *Cancer research* 67(15): 7275-83.

Chan, S.-K., Jaffe, L., Capovilla, M., Botas, J. and Mann, R. S. (1994) 'The DNA binding specificity of *Ultrabithora*x is modulated by cooperative interactions with *Extradenticle*, another homeoprotein.', *Cell* 78: 603-615.

Chang, C.-P., Jacobs, Y., Nakamura, T., Jenkins, N. A., Copeland, N. G. and Cleary, M. L. (1997) 'Meis proteins are major in vivo DNA binding partners for wild-type but not quimeric Pbx proteins.', *Molecular Cell Biology* 17: 5679-5687.

Chisa, J. L. and Burke, D. T. (2007) 'Mammalian mRNA splice-isoform selection is tightly controlled', *Genetics* 175(3): 1079-87.

Crijns, A. P., de Graeff, P., Geerts, D., Ten Hoor, K. A., Hollema, H., van der Sluis, T., Hofstra, R. M., de Bock, G. H., de Jong, S., van der Zee, A. G. et al. (2007) 'MEIS and PBX homeobox proteins in ovarian cancer', *European journal of cancer* 43(17): 2495-505.

Crist, R. C., Roth, J. J., Waldman, S. A. and Buchberg, A. M. (2011) 'A conserved tissue-specific homeodomain-less isoform of MEIS1 is downregulated in colorectal cancer', *PloS one* 6(8): e23665.

Cruz, C., Glavic, A., Casado, M. and de Celis, J. F. (2009) 'A gain-of-function screen identifying genes required for growth and pattern formation of the Drosophila melanogaster wing', *Genetics* 183(3): 1005-26.

de Navas, L. F., Garaulet, D. L. and Sanchez-Herrero, E. (2006) 'The ultrabithorax Hox gene of Drosophila controls haltere size by regulating the Dpp pathway', Development 133(22): 4495-506.

Dirk Geerts, N. S., Gerda Jorritsma, Rogier Versteeg (2003) 'The role of MEIS homeobox genes in neuroblastoma', Cancer Letters: 6.

Dong, P. D., Dicks, J. S. and Panganiban, G. (2002) 'Distal-less and homothorax regulate multiple targets to pattern the Drosophila antenna', *Development* 129(8): 1967-74.

Duboule D. (1992) 'The vertebrate limb: a model system to study the Hox/HOM gene network during development and evolution', *Bioessays* 14(6): 375-84.

Ebner, A., Cabernard, C., Affolter, M. and Merabet, S. (2005) 'Recognition of distinct target sites by a unique Labial/Extradenticle/Homothorax complex', *Development* 132(7): 1591-600.

Ekker, S. C., Jackson, D. G., von Kessler, D. P., Sun, B. I., Young, K. E. and Beachy, P. A. (1994) 'The degree of variation in DNA sequence recognition among four Drosophila homeotic proteins', *The EMBO journal* 13(15): 3551-60.

Foe, V. E. and Alberts, B. M. (1983) 'Studies of nuclear and cytoplasmic behaviour during the five mitotic cycles that precede gastrulation in Drosophila embryogenesis', *J Cell Sci* 61: 31-70.

Gehring, W. and Hiromi, Y. (1986) 'Homeotic genes and the homeobox', *Annual Review of Genetics* 20: 147-173.

Gilbert, S. F. (2003) *Developmental biology / Scott F. Gilbert*: Sinauer.

Glazov, E. A., Pheasant, M., Nahkuri, S. and Mattick, J. S. (2006) 'Evidence for control of splicing by alternative RNA secondary structures in Dipteran homothorax pre-mRNA', RNA Biol 3(1): 36-9.

Gonzalez-Crespo, S. and Morata, G. (1995) 'Control of *Drosophila* adult pattern by *extradenticle*.', *Development* 121: 2117-2125.

Gonzalez-Crespo, S., Abu-Shaar, M., Torres, M., Martinez, A. C., Mann, R. S. and Morata, G. (1998) 'Antagonism between extradenticle function and Hedgehog signalling in the developing limb', *Nature* 394(6689): 196-200.

Herranz, H. and Morata, G. (2001) 'The functions of pannier during Drosophila embryogenesis', *Development* 128(23): 4837-46.

Hisa, T., Spence, S. E., Rachel, R. A., Fujita, M., Nakamura, T., Ward, J. M., Devor-Henneman, D. E., Saiki, Y., Kutsuna, H., Tessarollo, L. et al. (2004) 'Hematopoietic, angiogenic and eye defects in Meis1 mutant animals', *The EMBO journal* 23(2): 450-9.

Hoey, T., Levine, M. (1998) 'Divergent homeo box proteins recognize similar DNA sequences in *Drosophilai*', *Nature* 332: 3.

Huang, H., Paliouras, M., Rambaldi, I., Lasko, P. and Featherstone, M. (2003) 'Nonmuscle myosin promotes cytoplasmic localization of PBX', *Molecular and cellular biology* 23(10): 3636-45.

Hyman-Walsh, C., Bjerke, G. A. and Wotton, D. (2010) 'An autoinhibitory effect of the homothorax domain of Meis2', *Febs J* 277(12): 2584-97.

Iotti, G., Longobardi, E., Masella, S., Dardaei, L., De Santis, F., Micali, N. and Blasi, F. (2011) 'Homeodomain transcription factor and tumor suppressor Prep1 is required to maintain genomic stability', *Proceedings of the National Academy of Sciences of the United States of*

America 108(29): E314-22.

Irimia, M., Maeso, I., Burguera, D., Hidalgo-Sanchez, M., Puelles, L., Roy, S. W., Garcia-Fernandez, J. and Ferran, J. L. (2011) 'Contrasting 5' and 3' evolutionary histories and frequent evolutionary convergence in Meis/hth gene structures', *Genome biology and evolution* 3: 551-64.

Jaw, T. J., You, L. R., Knoepfler, P. S., Yao, L. C., Pai, C. Y., Tang, C. Y., Chang, L. P., Berthelsen, J., Blasi, F., Kamps, M. P. et al. (2000) 'Direct interaction of two homeoproteins, homothorax and extradenticle, is essential for EXD nuclear localization and function', *Mechanisms of development* 91(1-2): 279-91.

Knoepfler, P. S. and Kamps, M. P. (1997) 'The Pbx family of proteins is strongly upregulated by a post-transcriptional mechanism during retinoic acid-induced differentiation of P19 embryonal carcinoma cells', *Mechanisms of development* 63(1): 5-14.

Kobayashi, M., Fujioka, M., Tolkunova, E. N., Deka, D., Abu-Shaar, M., Mann, R. S. and Jaynes, J. B. (2003) 'Engrailed cooperates with extradenticle and homothorax to repress target genes in Drosophila', *Development* 130(4): 741-51.

Kunwar, P. S., Starz-Gaiano, M., Bainton, R. J., Heberlein, U. and Lehmann, R. (2003) 'Tre1, a G protein-coupled receptor, directs transepithelial migration of Drosophila germ cells', *PLoS Biol* 1(3): E80.

Kurant, E., Pai, C. Y., Sharf, R., Halachmi, N., Sun, Y. H. and Salzberg, A. (1998) 'Dorsotonals/homothorax, the Drosophila homologue of meis1, interacts with extradenticle in patterning of the embryonic PNS', *Development* 125(6): 1037-48.

Lappin, T. R., Grier, D. G., Thompson, A. and Halliday, H. L. (2006) 'HOX genes: seductive science, mysterious mechanisms', *The Ulster medical journal* 75(1): 23-31.

LaRonde-LeBlanc, N. A. and Wolberger, C. (2003) 'Structure of HoxA9 and Pbx1 bound to DNA: Hox hexapeptide and DNA recognition anterior to posterior', *Genes & development* 17(16): 2060-72.

Lawrence, P. A. (1992) The making of a fly. Blackwell Scientific Publications.

Layalle, S., Volovitch, M., Mugat, B., Bonneaud, N., Parmentier, M. L., Prochiantz, A., Joliot, A. and Maschat, F. (2011) 'Engrailed homeoprotein acts as a signaling molecule in the developing fly', *Development* 138(11): 2315-23.

Leptin, M. (1995) 'Drosophila gastrulation: from pattern formation to morphogenesis', *Annual review of cell and developmental biology* 11: 189-212.

Lewis, E. B. (1978) 'A gene complex controlling segmentation in *Drosophila*', *Nature* 276: 565-570.

Mandhavan, M. S., H. A. (1977) 'Histological analysis of the dynamics of growth of imaginal discs y histoblasts nests during the larval development of Drosophila melanogaster', *Wilhem Roux Arch, devl biol* 183: 269-306.

Mann, R. S. (1995) 'The specificity of homeotic gene function', *BioEssays : news and reviews in molecular, cellular and developmental biology* 17(10): 855-63.

Mann, R. and Chan, S.-W. (1996) 'Extra specificity from *extradenticle*: the partnership between Hox and PBX/EXD homeodomain proteins.', *TIG* 7: 258-262.

Mann, R. and Affolter, M. (1998) 'Hox proteins meet more partners.', *Current Opinion in Genetics and Development* 8: 423-429.

McGinnis, W. and Krumlauf, R. (1992) 'Homeobox genes and axial patterning', *Cell* 68(2): 283-302.

McKnight, S. L. and Miller, O. L., Jr. (1976) 'Ultrastructural patterns of RNA synthesis during early embryogenesis of Drosophila melanogaster', *Cell* 8(2): 305-19.

Mercader, N., Leonardo, E., Azpiazu, N., Serrano, A., Morata, G., Martinez-A, C. and Torres, M. (1999) 'Conserved regulation of the proximodistal limb axis development by Meis/Hth', *Nature* 402: 425-429.

Miller, D. F., Rogers, B. T., Kalkbrenner, A., Hamilton, B., Holtzman, S. L. and Kaufman, T. (2001) 'Cross-regulation of Hox genes in the Drosophila melanogaster embryo', *Mechanisms of development* 102(1-2): 3-16.

Moens, C. B. and Selleri, L. (2006) 'Hox cofactors in vertebrate development', *Developmental biology* 291(2): 193-206.

Morgan, T. H. (1910) 'Sex Limited Inheritance in Drosophila', *Science* 32(812): 120-2.

Moskow, J. J., Bullrich, F., Huebner, K., Daar, I. R. and Buchberg, A. M. (1995) '*Meis1*, a *Pbx1*-

related homeobox gene involved in myeloid leukemia in BXH-2 mice.', *Molecular and cellular biology* 15(10): 5434-5443.

Nakamura, T., Largaespada, D. A., Lee, M. P., Johnson, L. A., Ohyashiki, K., Toyama, K., Chen, S. J., Willman, C. L., Chen, I. M., Feinberg, A. P. et al. (1996a) 'Fusion of the nucleoporin gene NUP98 to HOXA9 by the chromosome translocation t(7;11)(p15;p15) in human myeloid leukaemia', *Nature genetics* 12(2): 154-8.

Nakamura, T., Largaespada, D. A., Shaughnessy, J. D., Jr., Jenkins, N. A. and Copeland, N. G. (1996b) 'Cooperative activation of Hoxa and Pbx1-related genes in murine myeloid leukaemias', *Nature genetics* 12(2): 149-53.

Noro, B., Culi, J., McKay, D. J., Zhang, W. and Mann, R. S. (2006) 'Distinct functions of homeodomain-containing and homeodomain-less isoforms encoded by homothorax', *Genes & development* 20(12): 1636-50.

Otting, G., Qian, Y. Q., Billeter, M., Muller, M., Affolter, M., Gehring, W. J. and Wuthrich, K. (1990) 'Protein--DNA contacts in the structure of a homeodomain--DNA complex determined by nuclear magnetic resonance spectroscopy in solution', *The EMBO journal* 9(10): 3085-92.

Pai, C. Y., Kuo, T. S., Jaw, T. J., Kurant, E., Chen, C. T., Bessarab, D. A., Salzberg, A. and Sun, Y. H. (1998) 'The Homothorax homeoprotein activates the nuclear localization of another homeoprotein, extradenticle, and suppresses eye development in Drosophila', *Genes & development* 12(3): 435-46.

Parks, A. L., Cook, K. R., Belvin, M., Dompe, N. A., Fawcett, R., Huppert, K., Tan, L. R., Winter, C. G., Bogart, K. P., Deal, J. E. et al. (2004) 'Systematic generation of high-resolution deletion coverage of the Drosophila melanogaster genome', *Nature genetics* 36(3): 288-92.

Passner, J. M., Ryoo, H. D., Shen, L., Mann, R. S. and Aggarwal, A. K. (1999) 'Structure of a DNA-bound Ultrabithorax-Extradenticle homeodomain complex', *Nature* 397(6721): 714-9.

Peifer, M. and Wieschaus, E. (1990) 'Mutations in the *Drosophila* gene *extradenticle* affect the way specific homeo domain proteins regulate segmental identity.', *Genes and Development* 4: 1209-1223.

Peng, H. W., Slattery, M. and Mann, R. S. (2009) 'Transcription factor choice in the Hippo signaling pathway: homothorax and yorkie regulation of the microRNA bantam in the progenitor domain of the Drosophila eye imaginal disc', *Genes & development* 23(19): 2307-19.

Pineault, N., Abramovich, C. and Humphries, R. K. (2005) 'Transplantable cell lines generated with NUP98-Hox fusion genes undergo leukemic progression by Meis1 independent of its binding to DNA', *Leukemia* 19(4): 636-43.

Piper, D. E., Batchelor, A. H., Chang, C. P., Cleary, M. L. and Wolberger, C. (1999) 'Structure of a HoxB1-Pbx1 heterodimer bound to DNA: role of the hexapeptide and a fourth homeodomain helix in complex formation', *Cell* 96(4): 587-97.

Prochiantz, A. and Joliot, A. (2003) 'Can transcription factors function as cell-cell signalling molecules?', *Nature reviews. Molecular cell biology* 4(10): 814-9.

Rauskolb, C., Peifer, M. and Wieschaus, E. (1993) 'extradenticle, a regulator of homeotic gene activity, is a homolog of the homeobox-containing human proto-oncogene pbx-1', *Cell* 91: 171-83.

Rauskolb, C. and Wieschaus, E. (1994) 'Coordinate regulation of downstream genes by *extradenticle* and the homeotic selector proteins.', *EMBO Journal* 13(15): 3561-3569.

Rauskolb, C., Smith, K. M., Peifer, M. and Wieschaus, E. (1995) '*extradenticle* determines segmental identities throughout *Drosophila* development.', *Development* 121: 3663-3673.

Rieckhof, G. E., Casares, F., Ryoo, H. D., Abu, S. M. and Mann, R. S. (1997) 'Nuclear translocation of extradenticle requires homothorax, which encodes an extradenticle-related homeodomain protein', *Cell* 91(2): 171-83.

Rosales-Avina, J. A., Torres-Flores, J., Aguilar-Lemarroy, A., Gurrola-Diaz, C., Hernandez-Flores, G., Ortiz-Lazareno, P. C., Lerma-Diaz, J. M., de Celis, R., Gonzalez-Ramella, O., Barrera-Chaires, E. et al. (2011) 'MEIS1, PREP1, and PBX4 are differentially expressed in acute lymphoblastic leukemia: association of MEIS1 expression with higher proliferation and chemotherapy resistance', *Journal of experimental & clinical cancer research : CR* 30: 112.

Ryoo, H. D. and Mann, R. (1999) 'The control of trunk Hox specificity and activity by Extradenticle.', *Genes and Development* 13(13): 1704-1720.

Ryoo, H. D., Marty, T., Casares, F., Affolter, M. and Mann, R. S. (1999) 'Regulation of Hox target genes by a DNA bound Homothorax/Hox/Extradenticle complex.', *Development* 126: 5137-5148.

Salvany, L., Aldaz, S., Corsetti, E. and Azpiazu, N. (2009) 'A new role for hth in the early preblastodermic divisions in drosophila', *Cell cycle* 8(17): 2748-55.

Sanchez-Guardado, L. O., Irimia, M., Sanchez-Arrones, L., Burguera, D., Rodriguez-Gallardo, L., Garcia-Fernandez, J., Puelles, L., Ferran, J. L. and Hidalgo-Sanchez, M. (2011) 'Distinct and redundant expression and transcriptional diversity of MEIS gene paralogs during chicken development', *Developmental dynamics : an official publication of the American Association of Anatomists* 240(6): 1475-92.

Sanchez-Herrero, E., Casanova, J., Kerridge, S. and Morata, G. (1985) 'Anatomy and function of the bithorax complex of Drosophila', *Cold Spring Harb Symp Quant Biol* 50: 165-72.

Schneider, I. (1972) 'Cell lines derived from late embryonic stages of Drosophila melanogaster', *Journal of embryology and experimental morphology* 27(2): 353-65.

Sitwala, K. V., Dandekar, M. N. and Hess, J. L. (2008) 'HOX proteins and leukemia', *International journal of clinical and experimental pathology* 1(6): 461-74.

Spieker, N., van Sluis, P., Beitsma, M., Boon, K., van Schaik, B. D., van Kampen, A. H., Caron, H. and Versteeg, R. (2001) 'The MEIS1 oncogene is highly expressed in neuroblastoma and amplified in cell line IMR32', *Genomics* 71(2): 214-21.

Stamm, S., Ben-Ari, S., Rafalska, I., Tang, Y., Zhang, Z., Toiber, D., Thanaraj, T. A. and Soreq, H. (2005) 'Function of alternative splicing', *Gene* 344: 1-20.

Steelman, S., Moskow, J. J., Muzynski, K., North, C., Druck, T., Montgomery, J. C., Huebner, K., Daar, I. O. and Buchberg, A. M. (1997) 'Identification of a conserved family of Meis1-related homeobox genes', *Genome research* 7(2): 142-56.

Tan, D. and Walker, A. M. (2010) 'Short form 1b human prolactin receptor down-regulates expression of the long form', *Journal of molecular endocrinology* 44(3): 187-94.

Thorsteinsdottir, U., Kroon, E., Jerome, L., Blasi, F. and Sauvageau, G. (2001) 'Defining roles for HOX and MEIS1 genes in induction of acute myeloid leukemia', *Molecular and cellular biology* 21(1): 224-34.

Ui, K., Ueda, R. and Miyake, T. (1987) 'Cell lines from imaginal discs of Drosophila melanogaster', *In vitro cellular & developmental biology : journal of the Tissue Culture Association* 23(10): 707-11.

Van Dijk, M. A. and Murre, C. (1994) '*extradenticle* raises the DNA binding specificity of homeotic selector gene products.', *Cell* 78: 617-624.

Wang, G. G., Pasillas, M. P. and Kamps, M. P. (2006) 'Persistent transactivation by meis1 replaces hox function in myeloid leukemogenesis models: evidence for co-occupancy of meis1-pbx and hox-pbx complexes on promoters of leukemia-associated genes', *Molecular and cellular biology* 26(10): 3902-16.

Wernet, M. F., Labhart, T., Baumann, F., Mazzoni, E. O., Pichaud, F. and Desplan, C. (2003) 'Homothorax switches function of Drosophila photoreceptors from color to polarized light sensors', *Cell* 115(3): 267-79.

Winkelmann, J., Polo, O., Provini, F., Nevsimalova, S., Kemlink, D., Sonka, K., Hogl, B., Poewe, W., Stiasny-Kolster, K., Oertel, W. et al. (2007) 'Genetics of restless legs syndrome (RLS): State-of-the-art and future directions', *Movement disorders : official journal of the Movement Disorder Society* 22 Suppl 18: S449-58.

Wu, J. and Cohen, S. M. (1999) 'Proximodistal axis formation in the *Drosophila* leg: subdivision into proximal and distal domains by Homothorax and Distal-less.', *Development* 126: 109-117.

Xiong, L., Levchenko, A., Montplaisir, J., Riviere, J. B., Thibodeau, P., St-Onge, J., Gaspar, C., Desautels, A., Lesperance, P., Chouinard, S. et al. (2008) 'Genetic association studies of neurotensin gene and restless legs syndrome in French Canadians', *Sleep medicine* 9(3): 273-82.

Xiong, L., Catoire, H., Dion, P., Gaspar, C., Lafreniere, R. G., Girard, S. L., Levchenko, A., Riviere, J. B., Fiori, L., St-Onge, J. et al. (2009) 'MEIS1 intronic risk haplotype associated with restless legs syndrome affects its mRNA and protein expression levels', *Human molecular genetics* 18(6): 1065-74.

Yang, Y., Hwang, C. K., D'Souza, U. M., Lee, S. H., Junn, E. and Mouradian, M. M. (2000) 'Three-amino acid extension loop homeodomain proteins Meis2 and TGIF differentially regulate transcription', *The Journal of biological chemistry* 275(27): 20734-41.

Yeoh, E. J., Ross, M. E., Shurtleff, S. A., Williams, W. K., Patel, D., Mahfouz, R., Behm, F. G., Raimondi, S. C., Relling, M. V., Patel, A. et al. (2002) 'Classification, subtype discovery, and prediction of outcome in pediatric acute lymphoblastic leukemia by gene expression profiling', *Cancer cell* 1(2): 133-43.

Zeisig, B. B., Milne, T., Garcia-Cuellar, M. P., Schreiner, S., Martin, M. E., Fuchs, U., Borkhardt, A., Chanda, S. K., Walker, J., Soden, R. et al. (2004) 'Hoxa9 and Meis1 are key targets for MLL-ENL-mediated cellular immortalization', *Molecular and cellular biology* 24(2): 617-28.

VIII. APPENDICES

Resumen

En *Drosophila melanogaster* el gen *homothorax* (*hth*) tiene un papel importante durante el desarrollo; codifica para una proteína que pertenece a la familia conservada de factores de transcripción homeodominio-TALE (Bucle de extensión de Tres Aminoácidos, por sus siglas en inglés). Hth interacciona con Extradenticle (Exd), otro miembro de la familia TALE.

Aquí analizo las funciones de *hth* y su relación con *exd*. Hth presenta diversas funciones durante el desarrollo embrionario y larvario en *Drosophila*, entre las que se encuentran desde la subdivisión y especificación de partes del cuerpo hasta el ensamblaje de la heterocromatina. Las diversas funciones descritas para Hth residen en la complejidad de su *locus*, del cuál surgen seis diferentes isoformas.

Demuestro que distintas isoformas de Hth presentan diferentes patrones de expresión y son diferentes funcionalmente durante el desarrollo embrionario. Más aún, no todas las isoformas son capaces de translocar Exd al núcleo y la forma larga de Hth activa la transcripción de Exd. Los altos niveles de la proteína Exd facilitan su entrada al núcleo.

Este trabajo demuestra que *hth* es un gen complejo que no debe ser considerado como una unidad funcional simple. Los papeles de las diferentes isoformas residen posiblemente en sus distintos dominios proteicos y conformaciones, los cuales determinan, al final, sus interacciones con otras proteínas.

Introducción

La biología del desarrollo estudia los procesos morfogenéticos que llevan a la formación de un organismo. *Drosophila melanogaster* es uno de los organismos modelos más utilizados para estudiar la biología del desarrollo, por compartir el 60% de los genes con los humanos, por ser fácilmente manejable en laboratorio y por la existencia de numerosas técnicas de manipulación génica. El ciclo vital de *Drosophila* dura 10 días y, siendo un insecto holometábolo, pasa por un estadio embrionario, larvario y, tras una fase de pupa, llega a la edad adulta. La polaridad anteroposterior del embrión está finamente regulada por una cascada de genes, que termina con los homeóticos (*hox*), encargados de conferir la identidad segmental en todos los organismos. Los genes *hox* consiguen especificidad y afinidad en la unión al ADN gracias a la unión con cofactores, como Homothorax (Hth) y Extradenticle (Exd). Hth une Exd en el citoplasma a través de su dominio HM y lo transloca al núcleo, donde forman un complejo trimérico con una proteína Hox y se unen al ADN a través del homeodominio. Hth y Exd pertenecen a la familia de proteínas TALE, conservada en todas las especies e implicadas en graves enfermedades. Como cofactores de las proteínas Hox, el complejo Hth/Exd es necesario para la correcta identidad del sistema nervioso periférico embrionario (entre otras). Durante el desarrollo larvario el complejo Hth/Exd está implicado en la especificación de las regiones proximales de la pata y en el desarrollo de la parte posterior del *notum*. Hth es necesario también para el correcto ensamblaje de la heterocromatina pericentromérica en embriones tempranos. El *locus* de *hth* abarca una región de 100 kilobases, presenta 14 exones cortos separados por largos intrones. El splicing alternativo del ARN mensajero inmaduro de *hth* genera seis distintos ARN mensajeros. Las seis isoformas proteicas originadas incluyen tres largas, muy parecida entre ellas, que presentan el dominio HM y el homeodominio, una corta sin dominio HM, y dos cortas sin homeodominio.

Objetivos

1. Analizar la expresión y la distribución de las isoformas de Hth durante el desarrollo embrionario de *Drosophila* y en líneas celulares de *Drosophila*.

2. Intentar separar las funciones de las distintas isoformas durante el desarrollo embrionario de *Drosophila*.

3. Estudiar las interacciones regulatorias entre las diferentes isoformas de Hth.

4. Estudiar en más detalle la relación entre Hth y Exd.

Conclusiones

1. Los ARN mensajeros de *hth* se trancriben a distintos niveles en los embriones silvestres de *Drosophila*. Los más representados son los tres largos, seguidos por el hthRF y hthRG. Los niveles del hthRE no son detectables con las técnicas usadas.

2. Las isoformas proteicas de Hth muestran una distribución diferencial durante el desarrollo embrionario de *Drosophila*. La primera isoforma que se transloca al núcleo es la HthRF en el embrión blastodérmico temprano, cuando Exd aún es citoplasmático. Las isoformas con homeodominio entran en el núcleo mas tarde, poco antes de la extensión de la banda germinal. A partir de el estadio 9 la isoforma HthRF se reprime en los segmentos abdominales de el embrión.

3. Las isoformas de Hth muestran también una expresión diferencial en las líneas celulares de *Drosophila* S2 y DmD-8, sugiriendo que estas proteínas tienen distintas funciones dependiendo del tejido donde se expresan. La isoforma HthRE se localiza en al aparato de Golgi de las células S2 o DmD-8 y parece ser secretada.

4. Todas las isoformas de Hth parecen sufrir modificaciones postraduccionales en los embriones y en las líneas celulares, aunque no todas las isoformas presentan las mismas modificaciones en ambos sistemas, sugiriendo que las modificaciones postraduccionales tienen funciones importantes en el desarrollo.

5. Los mutantes Dfhth y PM1 presentan un fenotipo cuticular severo y los heterocigotos Dfhth/PM1 tienen un fenotipo más parecido al silvestre, sugiriendo que la combinación de las tres isoformas cortas podría restablecer la correcta segmentación.

6. La sobreexpresión de la isoforma HthRG en los mutantes Dfhth no rescata el fenotipo cuticular. La introducción da la HthRE provoca un leve rescate del fenotipo de los mutantes Dfhth. Las isoformas largas y la HthRF rescatan el fenotipo de los mutantes Dfhth.

7. En los mutantes Dfhth la isoforma HthRG, incluso al sobreexpresarse, no es capaz de translocar al núcleo. Las isoformas FlHth, HthRE y HthRF expresadas en los mutantes Dfhth permiten la translocación nuclear de Exd y de la isoforma HthRG.

8. La expresión ectópica de las isoformas largas y de HthRE en embriones silvestres provoca la represión de la isoforma HthRF. La expresión ectópica de la isoforma HthRE en embriones silvestres no tiene ningún efecto significativo sobre las isoformas con HD. La expresión ectópica de la HthRF en embriones silvestres no tiene ningún

efecto significativo sobre las demás isoformas.

9. La expresión ectópica de las isoformas largas en el dominio *spalt* del disco imaginal de ala y en el dominio *bric a brac* del disco imaginal de pata activa la transcripción de *exd*, llevando a un incremento de los niveles de la proteína Exd. Los altos niveles de Exd facilitan su translocación nuclear.